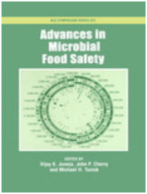

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# Advances in Microbial Food Safety

EDITED BY
Vijay K. Juneja, John P. Cherry
and Michael H. Tunick

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# Advances in Microbial Food Safety

In Advances in Microbial Food Safety; Juneja, V., et al.;
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# About the Cover

The picture on the front cover is a circular representation of genomic data from three sequenced *Listeria monocytogenes* genomes. Strains F2365 (serotype 4b; isolate from Hispanic cheese), F6854 (serotype 1/2a; turkey frankfurter isolate), and H7858 (serotype 4b; frankfurter isolate) are food isolates associated with human disease. Sequence data from these three strains have allowed genome comparisons between the 2 serotypes most frequently involved in food-related human illness and between strains belonging to 2 genomic divisions. These comparisons have led to the identification of serotype and strain specific genes that likely contribute to differences in pathogenicity and the ability of the organisms to grow in their respective environmental niches (Nelson et al., *Nucleic Acids Res.,* 32:2386–2395). The project was the collaborative work led by a team of researchers within the Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture at Wyndmoor, Pennsylvania and The Institute for Genomic Research (TIGR) in Rockville, Maryland. The sequence information is accessible via the Internet at www.tigr.org. These findings provide the framework for a host of laboratory experiments and computer data mining activities that in the years ahead will likely lead to better ways to manage the bacterium and lessen the occurrence and severity of listeriosis.

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# Advances in Microbial Food Safety

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# Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

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# Preface

Understanding the growth behavior of foodborne pathogens and their contamination of food matrices have dramatically increased and have continued at an unprecedented rate since the early 1990s. Microorganisms previously unknown or not known to be causes of foodborne illnesses and the reasons for their occurrence are continually being linked with documented outbreaks of illnesses. Foods identified and previously thought not to be involved in foodborne illnesses or believed to be infrequent sources of foodborne illnesses have been associated with outbreaks or sporadic episodes of sometimes fatal illnesses. The complexity of advancing pre-harvest, harvest and postharvest, including harvesting, handling, processing, and packaging, technologies increases the challenge to control all potential sources of microbial contamination. These food safety concerns are magnified because of consumer preferences for minimally processed quality, nutritious, and safe foods that offer convenience in availability and preparation. This includes processing fresh and ready-to-eat foods with these same properties. Hence, research institute scientists and engineers as well as those representing industries and federal, state, and local regulators, need to continually make advances in food preservation for pathogen control.

Major advances occurring in scientific and engineering principles and technologies contributing to the Hazard Analysis Critical Control Points (HACCP) system that are linked to microbial detection, their control or inactivation during processing and predictive modeling due to food safety research emphasize the need for a new comprehensive book. These observations, and our involvement through the years in food safety research, led us to the conclusion that such a book is timely. Accordingly, this symposium series book provides the reader with the latest research advances with insights into the microbiological safety of foods. The book is written by a team of experts who represent the best in the field of food safety. The basic knowledge about microbial adaptation to stress in food matrices is presented.

In Advances in Microbial Food Safety; Juneja, V., et al.;
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The rapid, genetic, and immunological biosensor-based methods for detecting foodborne microorganisms and their toxins are addressed. Included is quorum sensing as a key factor to microbial growth in foods. The problems of sampling the required sample enrichment processes prior to testing and the complexities of food environments impacting on pathogens are examined. Researchers explore different intervention approaches to kill, remove, or reduce pathogens in foods and offer quality, nutritious, safe, low-cost food products to consumers. Accordingly, recent developments in intervention strategies for control of foodborne microorganisms, microbial control–inactivation by traditional techniques, as well as by newer and novel nonthermal intervention methods such as ionizing radiation, pulse electric fields, high-pressure processing, use of natural antimicrobials., are addressed. The concept of predictive microbiology is a growing field that estimates the behavior of microorganisms in response to environmental conditions found in food matrices, including on farm to the table conditions is covered. Industry and regulatory perspectives and the challenges to ensure the safety of our food supply are presented. Every effort was made to write a comprehensive book on the current advances to making our food safe. We expect that the topics presented here will stimulate future innovative research studies.

It is necessary for the food industry and regulatory agencies to have personnel who are knowledgeable on available methods for detection and control or inactivation of microorganisms present in foods. This contributes to the development of regulations and optimization of HACCP. Currently, such information is presented in a variety of diverse sources, which are not always readily available. Accordingly, this book brings together these latest advances and should be of special benefit to those looking for a resource along with or in place of additional classroom training. This book is a valuable tool for those who are directly or indirectly involved in the production, handling, processing, distribution, and serving of food; control of hazards and spoilage of food products; inspection of food processing facilities; or doing research studies on microbial control or inactivation. Those in academic, industrial, and government institutions including federal, state, private, and local agencies, as well as food consultants, and lobbyists should find the book helpful in their work.

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This book evolved from the symposium *Advances in Microbial Food Safety*, which was sponsored by the Division of Agricultural and Food Chemistry, Inc. (AGFD) at the 228th National Meeting of the American Chemical Society in Philadelphia, Pennsylvania during August 22–26, 2004. Program planning and organization was led by scientists at the Eastern Regional Research Center (ERRC), Agricultural Research Service (ARS), U.S. Department of Agriculture. The ERRC is a leading research Center in postharvest microbial and chemical food safety research work in the Federal system. A notable feature of this symposium was the Sterling B. Hendricks Memorial Lectureship, an award sponsored by the ARS and presented annually at a joint session of AGFD and the ACS Division of Agrochemicals. The 2004 winner was Dr. Robert Buchanan of the Food and Drug Administration, whose award address "Uses and Limits of Microbial Testing" is included as Chapter 13 in this book.

We appreciate the excellent work of the authors and coauthors who were invited to contribute chapters in this book. The credit for making this book a reality goes to them. We as coeditors and the review team for the chapters especially appreciate sharing expertise with the contributors. We particularly thank the session organizers and we appreciate the support of AGFD for providing us with a forum for the symposium. We hope that this book will help in the design of future studies to advance new approaches to control foodborne pathogens and significantly contribute to technologies that decrease the incidence of bacterial foodborne illnesses due to foods.

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## Chapter 1

# Genomic and Proteomic Approaches for Studying Bacterial Stress Responses

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In the past the detection and analysis of pathogenic microorganisms in food has required the cultivation, isolation and identification of these organisms. This has been a time consuming endeavor. This approach has also not resolved the problem of those organisms that are "viable but non-culturable", that could not be identified by classical culture techniques. With the advent of genome sequencing, more molecular and global strategies for the identification of pathogenic organisms have become available. This presentation will describe and discuss molecular methods based on genomic and proteomic approaches to microbial identification. In addition, tools for analysis of gene expression in a community setting will be presented. Finally, strategies for the discovery of genes expressed during infection and pathogenesis will be considered.

Bacteria respond to varying environmental conditions by changing the expression of their genes. Most often this change in gene expression is co-ordinately controlled by a sigma factor that regulates the induction of a subset of genes in response to the change (*8, 11, 12*). This regulation is aimed at maintaining cellular homeostasis in the face of the changed environment. Although at first glance the study of bacterial stress responses does not seem to be of great utility, the information of how bacteria respond to stress is applicable to such diverse disciplines as medicine, pharmaceuticals and the food industry. In the food industry significant losses due to problems with food spoilage or pathogenic food-borne organisms are a reality. In order to minimize the risk of food contamination there is an urgent need to be able to detect the presence of spoilage and pathogenic organisms quickly and accurately. In addition, insights into bacterial responses to commonly used stress conditions used as food preservatives such as high salt and weak acids, can evaluate how spoilage and pathogenic organism will behave in these environments. In the field of infectious disease, study of bacterial responses to stress conditions within the host's body such as the acidic environment of the stomach and intestinal tract, will further our understanding of how pathogens evade these host defense systems.

In the past, such analyses were hard to perform due to the lack of fast and specific methods that targeted cellular responses to stress. With the advent of the genomics era, novel techniques have been innovated that expedite the analysis of global changes in gene expression in relatively short periods of time. These techniques have impacted scientific research and have allowed a wealth of information to be gained in such divergent fields as food safety and medicine. The insights gained will drive the development of improved methods for food preservation and food safety and will catalyze the discovery of new vaccine and antimicrobial technologies.

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## Why Study Bacterial Stress Responses?

Early studies on the physiology of bacterial species under stress conditions were carried out using exponentially growing laboratory cultures. Since then, many researchers have demonstrated that in natural habitats bacteria do not exhibit riotous, exponential growth., due partly to nutrient limitation and the build up of toxic metabolic by-products. In addition, the responses of bacterial species to stress often induces non-exponential growing phases such as stationary-phase in the bacterial population (*15*). Therefore, the responses shown by bacteria in exponential-phase are not generally the responses observed in stress induced situations, or in stationary-phase. In addition, one of the global responses to stress is the induction of cells that are either resistant to the stress or cells that enter a genetic program to form structures that protect them from the stress environment (*15*). Examples are *Escherichia coli* cells that show acid-resistance in low pH environments, or *Clostridium* and *Bacillus* species that form resistant spores in response to stress conditions. Therefore, the study of exponentially growing cells cannot begin to define and characterize the responses that occur in bacterial species when these organisms are confronted with a stressful, harsh environment. Very often the generalized stress response that is seen at the onset of exposure to stress in bacterial populations, is similar to responses observed in bacteria entering into stationary-phase (*8, 12*). The study of bacterial stress responses is very useful in a practical sense in food microbiology, infectious disease and in the study of the dynamics of natural populations. A detailed analysis of stress responses can be used to predict the behavior of a microorganism when faced with a particular environmental stress. These data can then be used to evaluate strategies for the preservation of food, evaluate the efficacy of specific drug therapies and for the analysis of bacterial populations in natural habitats.

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## The Rationale for Using Genomic Tools

With the advent of genome sequencing, more global strategies for the identification of microorganisms at the molecular level have become available. These strategies also lend themselves to analyzing changes in gene expression at the global, rather than local level. Best of all, genomic tools bypass the need for culturing organisms, since all that is required to perform these analyses is either genomic DNA or total mRNA isolated from the organisms under investigation (*26*). Several excellent commercial kits are available that cheaply, consistently and with high efficiency can be used for the routine extraction of genomic DNA or total mRNA from microbes and even microbial populations. These extraction methods coupled with genomic and proteomic-based tools have revolutionized the analysis of bacterial stress responses in environmental microbiology as well as in food microbiology, and in the study of microbial infection and pathogenesis. Three common problems associated with classic culture-based methods of detection and analysis are: the lack of suitable culture media for fastidious strains or species in low abiundance, the presence of viable but nonculturable organisms and the difficulty of analyzing global gene expression under stress conditions.

### The Lack of Appropriate Culture Media

In the past, the detection and analysis of pathogenic microorganisms in food has required the cultivation, isolation and identification of these organisms. Identification of the microorganisms usually requires the culturing of these organisms on selective media combined with several metabolic tests. This is a tedious and time consuming endeavor, that sometimes requires several weeks for a definitive answer. Culture-based methods can also require multiple enrichment steps to enable the isolation of those organisms present in small numbers or with fastidious growth requirements. A problem with culture-based methods is the lack of suitable growth media to support the growth of all but a few species. The number of existing microbial species is roughly estimated at $10^5$-$10^6$. Kaeberlein et al. (*13*) argued that only a few thousand species have been isolated in pure culture because very few microbes isolated from the natural environment grow on nutrient media in the laboratory. Culture-based techniques therefore, have many drawbacks, and do not quickly and efficiently aid in bacterial isolation and characterization. Moreover, culture-based methods do not easily allow the growth of fastisious organisms.

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## Viable but Non-Culturable Organisms

Viable but non-culturable (VBNC) organisms do not grow on the usual media used for the selection of most microbes, and thus can be missed during culture-based detection processes (*5*). Rice et al. (*23*) have defined the viable but non-culturable state as a physiological state having a specific block that prevents VBNC organisms from dividing and growing on media which normally supports their growth. Food-borne pathogenic microorganisms like *Vibrio vulnificus* can show the VBNC response, while still maintaining an infectious state (*23*). Since normal culture techniques will not be successful in isolating organisms in a VBNC state, these organisms still possess the ability to infect and cause disease in the host. VBNC organisms are a serious threat to human health and safety. In particular, those VBNC organisms that are carried in food (e.g. *V. vulnificus*) pose a serious threat to food safety and the health of the public.

## Analysis of Global Gene Expression

A third problem arises when trying to detect genes expressed in a particular organism under different growth or environmental conditions, or under normal and stress conditions. These experiments have usually involved culturing the organism under normal versus stress conditions, and then employing either Northern blot analysis or reverse-transcription polymerase chain reaction (RT-PCR) to detect changes in gene expression. Under these experimental conditions, only a relatively few genes can be analyzed by either Northern blots or RT-PCR. An extensive review on PCR-based techniques is presented in another chapter of this book by Liu and Fratamico, and therefore, PCR-based methods will not be discussed in this section. The situation is more complex when one is trying to elucidate patterns of gene expression in a mixed microbial population. Again, culture-based techniques will not represent all the organisms present with a high degree of fidelity. A more accurate assessment of the organisms present and their gene expression profiles can be obtained using techniques that use molecular tools to target genetic loci.

## Genome-Based Methods

Genome-based methods encompass the techniques of DNA microarrays and proteomics (*26, 27*). DNA microarrays rely on the hybridization of DNA or RNA to selected oligonucleotides that represent the genome of the organism. These oligonucleotides are chosen by analysis of published genome sequence data. Proteomics uses two dimesional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2-D SDS-PAGE) to separate the total proteins from a microbial species or population, followed by mass spectrometry of the isolated proteins for identification. Genomic and proteomic techniques do not require intact and viable microorganisms, bypassing the need for culturing the organisms under analysis.

### DNA Micro-Array Based Detection

DNA microarrays are fast becoming a very accurate technique for analysis of global gene expression, and for the detection of species in a microbial population. Essentially a glass surface, usually a slide, is spotted with a defined set of oligonucleotides that represent an entire genome or a subset of genes in a genome. The oligonucleotides are synthesized after careful analysis of the genome sequences of the organsism(s) and should be representative of the genome of the organisms under study. Several methods are available for the preparation of the microarray slide and these have been excellently reviewed by van Hal et al. (*26*). For analysis of global gene expression at the transcriptional level, total mRNA is extracted from the organism(s), labeled with a fluorescent tag, and the mRNA hybridized to the oligonucleotide containing slide or "chip." Detection of binding of specific mRNA to specific oligonucleotides is carried out by a microarray reader, which can be a charge-coupled device (CCD) camera, non-confocal laser scanner or confocal laser scanner. Commercial data acquisition and handling software are available for the analysis of the data generated by a microarray. In studying the response of genes to stress conditions, it is usual for two sets of fluorescently labeled mRNA to be hybridized separately to two DNA microarrays. One set of mRNA is isolated from organisms grown under standard conditions, and the second set is total mRNA

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isolated from the same organism that has been subjected to the stress condition under study (e.g. heat shock, osmotic stress, cold shock). A comparison of the intensity of the fluorescent signals generated from both microarray sets reveals genes that are up-regulated or down-regulated in response to the stress condition. For detection of microorganisms, the microarray is hybridized with labeled genomic DNA fragments isolated from the sample. Analysis of the signals obtained with genomic DNA is similar to that described for mRNA.

DNA microarray technology has been used in numerous examples to analyze gene expression. It has been used to detect the expression of "foreign" genes in genetically modified plants, to study genes expressed in response to hydrogen peroxide (oxidative stress) in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (*16*), and to analyze genes expressed in the global stress response of the gram positive bacterium *B. subtilis* (*21*) to name but a few. The potential of this technique in the fields of infectious disease and pathogenesis are enormous, since genes that are specifically expressed during infection and disease can be identified by side-by-side comparisons with genes that are expressed by the pathogen in the free-living state.

A limitation of this technique is that it is expensive and requires that the genome sequences of the organisms under study should be available for designing the oligonucleotides for the microarray.

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## Proteomics

Proteomics-based techniques are used to determine the protein expression profile of an organism under given conditions (*10, 20, 22*). This technique is empirically more challenging than that of DNA microarrays, since it requires the extraction of total proteins in the cell. The profile of the extracted proteins should represent all protein classes present in the cell both qualitatively and in abundance. Proteins are then separated by two dimensional SDS-polyacrylamide gel electrophoresis, and the separated proteins identified by mass spectroscopy coupled with N-terminal sequencing of the mass spectroscopy generated peptides. The use of this technique is not as widespread as that of DNA microarrays due to the challenges associated with the purification and separation of the complex mixtures of proteins found in cell extracts. This technique has been used to study the cold adaptation of *E. coli* (*19*) and as a tool to improve the "substantial equivalence" of genetically modified organisms (*6*). Substantial equivalence refers to whether a food from a genetically modified organism corresponds totally from a digestive point of view, to the traditional one, and is a major issue in the controversy plaguing the use of transgenic organisms as sources of food.

Proteomics has also been used to analyze the proteins released during the ripening of Emmentaler cheese. In an innovative study, Gagnaire et al. (*7*), used proteomics to prepare a reference map of the different groups of proteins found within cheese. These authors were able to categorize the proteins found in the cheese into five classes: those involved in proteolysis, glycolysis, stress response, nucleotide repair and oxidation-reduction. In addition, information was obtained regarding the peptidases released into the cheese during the ripening process. This study enabled Gaganire et al. to differentiate between the various casein degradation mechanisms present, and to sugest that the streptococci within the cheese matrix are involved in peptide degradation and together with the indigenous lactobacilli contribute to the ripening process. Using proteomics these authors were able to derive a greater understanding of the microbial succession involved in the ripening of Emmentaler cheese, which information could not have been obtained using other protein separation technique. This example illustrates the power of proteomics as a tool for analyzing the composition of a complex mixture of proteins and peptides.

The strength of genome-based technology relies on the accuracy and validity of the genome sequence information available (*4*). Very often, however, the information obtained from genomics and proteomics does not assign a putative function to the genes and proteins identified. If the genes/proteins identified by the genomics-based approaches have been previously well characterized, then it gives the researcher a starting point with which to set up future investigations. But, if the gene or protein has only been annotated as a putative open reading frame without a function attributed to it, then this information does not yield any clues to the possible function of the gene/protein. The correlation between a gene/protein sequence and function in the organism has to be carried out by basic empirical research.

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## Techniques for Determining the Function of Identified Genes

The identification of genes and proteins that are regulated by a particular stress response using genomic methods has to be correlated or, at least, associated with a particular function for the genomic information to have value. Techniques of classic microbial genetics are used to identify and characterize the function of selected genes. In microbial genetics, gene function is usually identified by creating, isolating and identifying mutants in the signaling pathway or cellular process under study that correlates to a specific phenotype. Phenotypes that are selected for can be acid-resistance, high-salt resistance or avirulent mutants of pathogenic organisms. An in-depth study of the aberrant mutant phenotype is then carried out to discover where in the process the precise malfunction occurs. The malfunctioning gene is then identified and the correct function attributed to it. The power of microbial genetics is not only in the ability to create mutations by genetic or chemical means (mutagenesis), but also in the ability to identify the mutants (selection) and to recover the genetic site of the disruption. Transposon mutagenesis is a commonly used genetic technique for the *in vitro* or *in vivo* creation of mutant phenotypes.

## Transposon mutagenesis

In this type of mutagenesis, a transposon is delivered by electroporation, mating or conjugation to the wild-type cells of the organism of interest and allowed to randomly "hop" (or transpose) into any locus in the organism's genome. The transposition event is catalyzed by the enzyme transposase. By transposing into a gene locus, the transposon creates a mutation in that gene by inserting into it. The insertion of the transposon generally inactivates the gene, such that the mutant created in this way has a loss of that particular gene's function. If the transposon locates into the regulatory regions of the gene, it can also cause up-regulation of the gene and create a situation where there is excess of that gene product in the cell. In either case, there is imbalance in the amount of the gene product in the cell that consequently causes a mutant phenotype. In the best case scenario, the mutant phenotype is an easily detectable and visible one, allowing for the easy isolation of these mutants. Most often, a clear, visible phenotype is not available. In these instances, many strategies have been described for the identification and isolation of the desired mutant phenotype. Discussed below are two approaches (signature-tagged mutagenesis and the negative selection method) that allow the identification and retrieval of aberrant genes in a pathway. Both methods employ negative selection strategies, that are so named because the identified cells are mutant in nature, allowing for easy retrieval of the mutant cells.

Insertion of the transposon into genomic DNA can be done either *in vitro* or *in vivo*. Epicenter Technologies (www.epicentre.com/transposome.asp) has developed a commercially available transposon mutagenesis system that can be used with extracted genomic DNA or with intact, viable cells. If extracted genomic DNA is used as the substrate for transposon activity, the transposon inserted DNA can be amplified in *E. coli* before it is introduced into viable cells with selection for the antibiotic marker on the transposon. If viable cells are used, the transposon is introduced into the cells by electroporation and after insertion the transposase enzyme is inactivated by salt. This method can only be used with those bacterial cells that allow electroporation for the introduction of DNA fragments. In systems where the introduction of DNA by electroporation is not an option, transposons can be introduced into cells via conjugation. Historically, a number of transposon mutagenesis schemes have been developed

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for both gram positive and negative bacteria prior to the advent of the commercial kit, and can be used with high rates of success.

### *Signature-Tagged Mutagenesis*

This method of mutagenesis and selection (*17*) has been used successfully to identify genes involved in the virulence process in *Salmonella* serovar Typhimurium, *V. cholerae* and *Klebsiella* pneumoniae (*9*). This technique combines insertional mutagenesis with the negative selection *in vivo* of avirulent or attenuated pathogenic strains. Transposon mutagenesis is used to generate a bank of mutant bacteria. This pool of mutant bacteria is then introduced into the host animal model. After incubation in the host, the bacteria are isolated and the signatures are amplified (Figure 1) to identify those tags that were lost due to death of the avirulent bacterial cells within the host. These dead bacteria represent those cells that were unable to infect the host successfully, due to the transposon insertion into a genetic locus essential for virulence. The "lost" tags can be identified by hybridization of the recovered tags to the master collection of bacteria containing all of the initially generated transposon tagged loci. Those bacteria that do not hybridize to the recovered tags and that represent the "lost" tags, contain transposon insertions in genes required for virulence. The transposon insertion site can be easily identified by locating the transposon itself, the DNA region containing the transposon isolated and the inactivated gene identified by DNA sequencing around the insertion site. The genes thus identified are required for the virulence process.

### *Negative Selection Strategy Using the codA Gene*

This genetic selection scheme was originated, developed and tested in our laboratory (*2*). The scheme is based on the fusion of a inducible promoter to the cytosine deaminase (*codA*) gene of *E. coli* (Figure 2). The promoter of choice used was the high-light regulated *psbDII* promoter (*1*) from the free-living cyanobacterium *Synechococcus elongatus*. The *psbDII* promoter was fused to the *codA* gene such that all regulatory information (promoter sequences and the ribosomal binding site) were from the *psbDII* gene. The construct was then introduced by transformation into *Synechococcus* cells, and homologously recombined into a neutral site in the *Synechococcus* chromosome. Neutral sites are regions of the *Synechococcus* chromosome where genetic constructs can be recombined without any ill effects on the growth and viability of the organism (*3*). The resulting strain was then mutagenized using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MMNG) to generate random, point mutations in the

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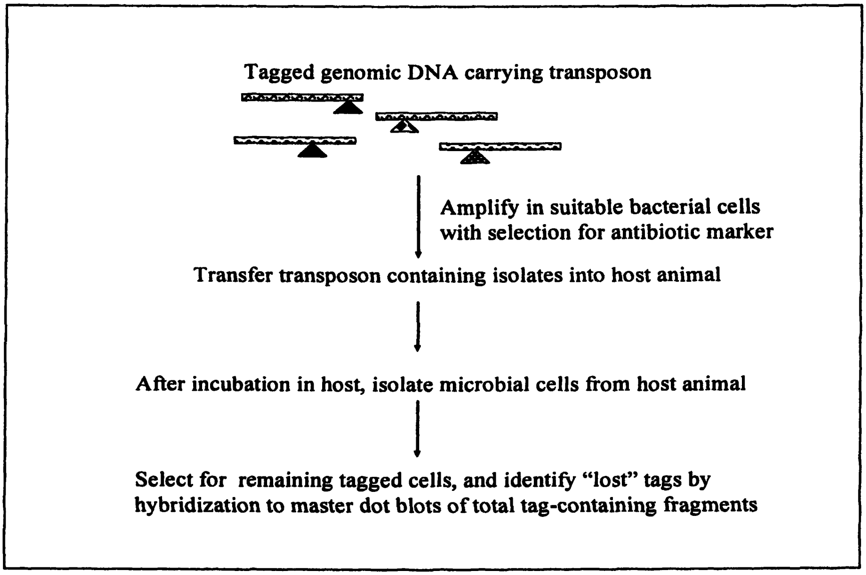
Tagged genomic DNA carrying transposon
Amplify in suitable bacterial cells with selection for antibiotic marker
Transfer transposon containing isolates into host animal
After incubation in host, isolate microbial cells from host animal
Select for remaining tagged cells, and identify "lost" tags by hybridization to master dot blots of total tag-containing fragments

*Figure 1. Diagrammatic representation of genes that are expressed in vivo using signature tagged mutagenesis.*

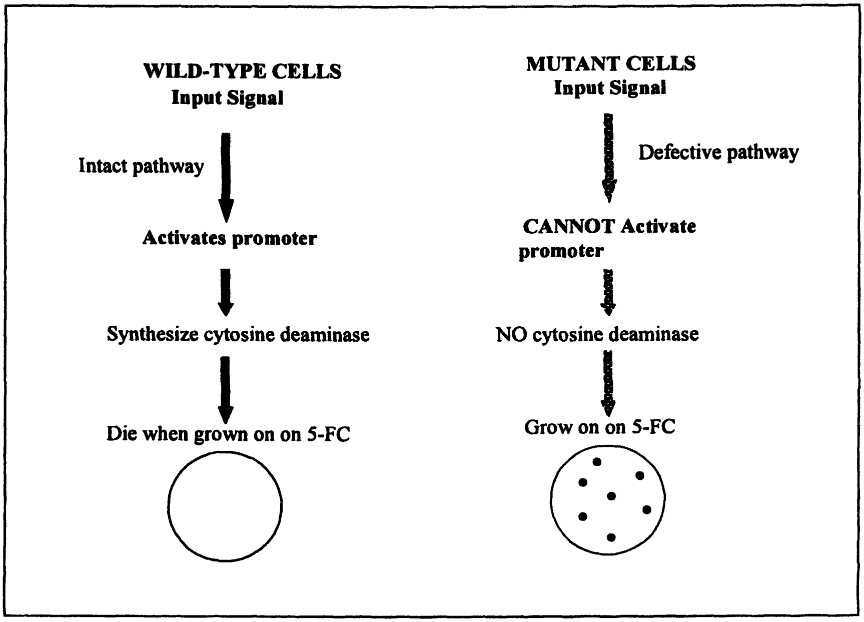
WILD-TYPE CELLS
Input Signal
Intact pathway
Activates promoter
Synthesize cytosine deaminase
Die when grown on on 5-FC
MUTANT CELLS
Input Signal
Defective pathway
CANNOT Activate promoter
NO cytosine deaminase
Grow on on 5-FC

*Figure 2. Cytosine deaminase-based negative selection scheme for the isolation of mutants.*

chromosome, and the mutagenized cells grown on medium containing the selective agent 5-fluorocytosine.

The enzyme cytosine deaminase, protein product of the *codA* gene, converts 5-fluorocytosine into the toxic product 5-fluorouracil (*24*). *Synechococcus* cells do not contain an intrinsic *codA* gene, and this gene is an excellent selective tool in this scheme. The scheme functions conceptually as follows. Cells that contain an intact high-light signaling pathway that regulates the *psbDII* promoter will express the *codA* gene in high light. Expression of this gene results in the synthesis of the enzyme cytosine deaminase which will convert 5-fluorocytosine to the toxic metabolite 5-fluorouracil, and cells growing on this substrate will die. However, cells in which the *psbDII* high-light pathway is defective due to a mutation in the pathway, will NOT express the *codA* gene and will survive when grown on 5-fluorocytosine due to their inability to convert this chemical into its toxic product. These cells will carry the desired mutations in the *psbDII* pathway. To identify the site of mutation, the resulting mutants can be individually "rescued" with genomic DNA fragment from wild-type *Synechococcus*. Rescued cells will display the wild-type ability to express the *codA* gene and will die when challenged with 5-fluorocytosine. This challenge can be used as cofirmation that the mutant phenotype has been rescued by the wild-type genomic DNA fragment.

This negative selection scheme can be used where a clear, visible mutant phenotype is not available for the easy selection of mutants. The *codA* marker can be used in mammalian cells (*14, 25*) and in bacterial cells that lack a *codA* gene, or where the native *codA* gene has been disabled prior to use in this scheme. We tested this scheme with mutants that had been created by chemical mutagenesis. Other mutagenesis methods also lend themselves for use with this selection scheme. If transposon mutagenesis is used, the site of mutation can easily be located by isolating the region of transposon insertion in the genome.

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## *In vivo* Expression Technology (IVET)

This method has been used to isolate genes that are expressed in the animal host during infection, but are not expressed in the free-living pathogenic organism. Mahan et al. (*18*) used this technique to isolate genes expressed during the infection of mice with the bacterium *Salmonella* serovar Typhimurium. Randomly generated *Salmonella* genomic DNA fragments were fused upstream of promoterless, tandomly arranged *purA* and *lacZ* genes, and the construct transformed into *E. coli*. The *purA* gene is required for purine biosynthesis in the bacterium and the *lacZ* gene (when activated) serves as a reporter gene. The constructs were then transferred by conjugal mating from *E. coli* to a *purA* defective *Salmonella* strain, and integrated into the *Salmonella* chromosome by homologous recombination. The resulting *Salmonella* cells were used to infect the animal host, the mouse. *Salmonella* cells were incubated in the mouse host for 2-3 days, to allow for selection of all *Salmonella* cells that had a *purA*$^+$ phenotype. The *purA*$^+$ phenotype would only have occurred if the genomic DNA fragment cloned upstream of the *purA* gene in the construct, contained a promoter that was activated upon infection of the mouse host. Any *Salmonella* cells that were *purA* defective would not be viable in the mouse host. Bacterial cells were recovered from the spleen of the host animals, and plated on indicator medium for *lacZ* gene expression. Mahan et al. *(18)* were interested in those *Salmonella* genes that were expressed in the host, but not in the free-living state on a laboratory medium. Thus, they isolated bacterial colonies that were Lac$^-$ and were white not blue in coloration on the indicator plate, since cells that were Lac$^+$ on laboratory medium will contain DNA fragments that activated *lacZ* (and *pur*A) expression in the free-living state. These authors were able to successfully identify genes associated with *Salmonella* virulence in the mouse host.

The advent of genomics-hased techniques has revolutionized the analysis of bacterial gene expression in response to stress. For maximum impact and information, these techniques have to be coupled with classical microbial genetics methods to yield critical insights on bacterial stress responses. These data will greatly impact the fields of food safety, infectious disease and the design of antimicrobial technologies.

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## Chapter 2

# Overview of Rapid Methods for the Detection of Foodborne Pathogens and Toxins

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Analysis of foods for pathogens and toxins is a standard practice that has been done using mostly conventional microbiological assays. Advances in technology however, changed food testing procedures by introducing "Rapid Methods" that use antibodies, nucleic acids, special substrates, etc, that can detect these contaminants faster, simpler and with more sensitivity and specificity than conventional tests. As a result, they are ideal for screening foods for the presence or absence of pathogens or toxins. But the complexities of foods continue to be problematic and some culture enrichment or extraction is still required prior to analysis. Positive rapid method results are often regarded as presumptive and require confirmation. Also, assay efficiencies may vary depending on foods, hence methods need to be comparatively evaluated or validated before routine use. More sensitive and faster assays are being developed, but the complexity of foods continues to present challenging problems.

Microbiological testing is a standard practice used by the industry and regulatory agencies to monitor contamination in foods. But testing foods for pathogens or toxins is a challenging task due to the variations in food composition and matrices. To overcome these problems, conventional methods use media to enrich, select, isolate and identify pathogens in foods. Similarly, in microbial toxin testing, extractions and concentration steps had to be used prior to detection by serological or animal assays.

Advances in biotechnology introduced new technologies, which had a tremendous impact on food testing methods. These assays, collectively known as "Rapid Methods" uses antibodies, nucleic acids, specialized substrates and automation, to detect pathogens and toxins specifically, sensitively and rapidly. However, in testing foods, they are not free of limitations, as rapid methods remain susceptible to food matrix problems, hence, necessitating enrichment and sample preparation procedures, which compromises speed of analysis.

Foods come in many physical forms (powder, liquid, gel, solid, semi-solid, etc) and their composition is even more varied as they are made up of various combinations of ingredients like carbohydrates, proteins, fats, oils, and chemicals, some of which can interfere with mixing, resulting in heterogenous samples. Compounded by the fact that bacteria are not uniformly distributed in foods, an aliquot tested may not necessarily be representative of the overall sample, so the result may be irreproducible.

In addition to matrix problems, normal microflora that are found in many foods and especially at high levels ($10^8$ cells/g) in raw foods, can interfere with the detection of pathogens, which are found at much lower levels but can still cause illness. Interference is further enhanced if food processing procedures have stress-injured the pathogens and they may be out competed by flora during enrichment.

To overcome these problems, sample preparation steps had to be modified or adapted for specific foods and samples had to be enriched to resuscitate injured cells, suppress normal flora and to growth-amplify the pathogens prior to detection. Normal flora poses less problems in toxins testing but the complexity of matrices, low toxin levels and processing, which can denature toxins and affect their antigenicity, are of concern, hence, extraction and concentration steps are required prior to detection.

Although conventional methods are most often used in food testing, and long regarded as the gold standard, they are labor intensive and time-consuming and therefore, inadequate for making quick assessments on the microbiological quality and safety of foods.

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## Rapid Methods

### Origin and Definition

The emergence of rapid methods is linked directly to biotechnology, which in turn originated from the wealth of knowledge derived from years of basic molecular research (*1*). Although the term "rapid methods" has only existed in the literature for the past 20 years or so, the speed with which rapid methods developments took place surprised many. In 1981, international experts were invited to the Delphi Forecast to speculate on the future technologies that will be used for the detection of bacteria in foods (*2*). Most of the technologies predicted by the panel were accurate and are used today, but the potential application of antibodies and nucleic acids as diagnostic tools were not predicted by the panel, and yet these two technologies came to dominate the area of rapid diagnostic methods.

Development of rapid methods is currently a competitive industry that enjoys popularity and interest worldwide. There is however, no set definition of what is a rapid method and to come up with such a definition is probably not feasible, as the term "rapid" is subject to interpretation. As a result, "rapid methods" includes a large group of assays that uses various technologies and ranges from tests that can give results in minutes to those that simply shortens conventional assay procedures, which in some cases take several days or even up to a week to complete (*3*).

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## Formats and Technologies

Because of the subjective definition of "rapid", the assay formats and technologies used in rapid methods are extremely diverse. But regardless of format, most rapid methods used for detecting bacterial pathogens in foods still require culture enrichment and the assays for toxins still need extraction or concentration. So, the assay may be rapid but the testing of foods is much slower due to the sample preparation requirements. For example, miniaturized biochemical tests, including automated and other identification tests, can rapidly identify bacteria, sometimes within 4 hrs. However, the isolate has to be a pure culture and the isolation procedure remains conventional, requiring media to grow, select and isolate the colonies, which can take several days.

Other rapid methods are modification of conventional methods but are less labor intensive and shortens analysis time. For instance, some assays use disposable cardboards with hydratable selective media so that preparations and disposals before and after testing are greatly simplified. Some assays use specialized substrates in media and measure changes in optical density or other metabolic products from the growth of specific bacteria. Yet, others use fluorogenic or chromogenic substrates, which cause color changes in colonies to provide presumptive identification of bacteria that express specific enzymes (*4, 5*). All these assays simplify and shorten test times but, continue to require growth incubation.

Recently, the use of adenosine triphosphate (ATP) to measure total bacterial load is another concept that has been introduced to food testing (*6*). ATP assays do not require culture enrichment, hence, provide a quick indication on the sanitary quality of a food processing environment or a product within minutes. But, since all living cells have ATP, procedures have to be included to separate bacteria from yeast or mammalian cells. Similarly, because ATP assays could not differentiate between bacterial species, they were used solely for estimating total bacterial counts. But, some ATP assays have been modified by using specific antibodies and immunomagnetic separation to selectively capture target bacteria from foods then, used bacteriophages to lyse the specific bacterial hosts to release ATP for measurement.

The two technologies that had the most impact on testing methods include DNA and antibody analysis, and these assays dominate the field of rapid methods (*7*). The three prevalent DNA assay formats are probe, cloned bacteriophages and PCR (Table I). Probe assays usually target ribosomal RNA (rRNA) to take advantage of the fact that the higher copy number of bacterial rRNA provides a naturally amplified target and affords greater sensitivity. To detect the specific hybridization of DNA probe to their targets, some assays couple their probes with a chemiluminescent label for detection via fluorescence, but others use biotin for detection by strepavidin- antibody conjugates using enzyme linked immunosorbent assays. Some DNA probe assays are designated for use solely for the identification of pure cultures of bacteria, but others are used for testing for the presence of pathogens in food enrichment cultures.

The specific interaction of phage with its bacterial host has also been used to develop assays for detecting pathogens. Two examples are ice nucleation (*8*) and bioluminescence (*6*), where phages cloned with *ina* and *lux* genes, respectively, are used as reagents to test food enrichment cultures. Since phages only infect specific bacterial hosts, the detection of phenotypes expressed from the genes cloned into the phage is indicative that the particular bacteria were present in the sample.

Polymerase chain reaction (PCR) is an extremely powerful tool that uses enzymes and target-specific oligonucleotide primers to exponential amplify a gene sequence in a short time. PCR has been a commonly used research tool in the laboratory for many years, but the numerous manipulations and the use of agarose gel electrophoresis to visualize amplified products, were not user-

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**Table I. Patial Listing of DNA-Based Rapid Methods for Bacteria**

| *Bacteria* | *Assay* | *Format*[a] | *Company* |
|---|---|---|---|
| *Campylobacter* | GENETRAK | probe | Neogen |
| | AccuProbe | probe | Gen-Probe |
| | Probelia | PCR | Sanofi Pasteur |
| *Clostridium botulinum* | Probelia | PCR | Sanofi Pasteur |
| *Enterobacter sakazakii* | BAX | PCR | Qualicon |
| *Escherichia coli* | GENETRAK | probe | Neogen |
| | Genevision | rtPCR | Warnex |
| *E. coli* O157:H7 | BAX | PCR | Qualicon |
| | Probelia | PCR | Probelia |
| | Genevision | rtPCR | Warnex |
| | TaqMan | rtPC | Perkin Elmer |
| | GENETRAK | probe | Neogen |
| | AK-Phage | IMS/ATP | Alaska Diag. |
| *Listeria* spp. | GENETRAK | probe | Neogen |
| | BAX | PCR | Qualicon |
| | OligoScan | probe | MicroTech LLC |
| | RABIT | probe | Don Whitley Sci |
| | Genevision | rtPCR | Warnex |
| | AK-Phage | IMS/ATP | Alaska Diag |
| *L. monocytogenes* | Probelia | PCR | Sanofi Pasteur |
| | BAX | PCR | Qualicon |
| | AccuProbe | probe | Gen-Probe |
| | Foodproof | PCR | Biotecon Diag |
| | GENETRAK | probe | Neogen |
| | Genevision | rtPCR | Warnex |
| | AK-Phage | IMS/ATP | Alaska Diag. |
| | LightCycler | rtPCR/probe | Roche |
| *Salmonella* | GENETRAK | Probe | Neogen |
| | BAX | PCR | Qualicon |
| | BIND | phage | Idexx |
| | Probelia | PCR | Sanofi Pasteur |
| | Genevision | rtPCR | Warnex |
| | TaqMan | rtPCR | Perkin Elmer |
| | LightCycler | rtPCR/probe | Roche |
| | Foodproof | PCR | Biotecon Diag |
| | RABIT | probe | DonWhitley Sci |
| | AK-Phage | IMS/ATP | Alaska Diag. |
| *Shigella* | Foodproof | PCR | Biotecon Diag |
| *Staphylococcus aureus* | GENETRAK | probe | Neogen |
| | AccuProbe | probe | Gen-Probe |
| | Genevision | rtPCR | Warnex |
| *Yersinia enterocolitica* | GENETRAK | probe | Neogen |

[a] Probe: DNA probe; PCR: polymerase chain reaction; rtPCR: real-time PCR; IMS/ATP: immunomagentic separation Adenosine triphosphate.

friendly enough for a food diagnostic setting. However, advances in instrumentations enabled automation of PCR assays and furthermore, the introduction of real-time PCR assays that not only enabled even faster amplification, but also provided real-time results, has greatly increased the potential of using PCR to detect for pathogens in foods (*9, 10*). Several PCR and real-time PCR assays using various detection systems, such as Sybrgreen, FRET probes, TaqMan, and molecular beacon, are already commercially-available for testing for pathogens in foods. Furthermore, PCR is a critical component of next generation assays such as microarray that are being developed, which enable simultaneous detection of multiple genes on a single chip (*11, 12*).

PCR can theoretically amplify a copy of DNA a million fold in a few hours; hence this technology has the potential to eliminate the need for enrichment to growth-amplify bacteria (*13*). But, numerous attempts to use PCR in food testing have found that many foods contained substances that inhibited or interfered with PCR (*13, 14*). As a result, the sensitivity achievable by PCR with pure cultures, were often reduced when testing foods and that some cultural enrichment was still required prior to PCR analysis.

The specific binding of antibody to antigen and the simplicity of this interaction has facilitated the design of many assays and formats and they comprise the largest group of rapid methods used in food testing (*15*).

Latex agglutination (LA) is the simplest antibody test, where antibody-coated colored latex beads or colloidal gold are used to test cell suspensions of pure bacterial cultures. The presence of specific antigens is indicated by clumping and the reaction takes less than a minute, so it is a very rapid and useful serological typing tool. Reverse passive latex agglutination (RPLA) is a variation of LA; the main difference being that in LA, the antigens (cells) are insoluble, whereas in RPLA, the antigens (proteins) are soluble, so it is used mostly in testing for toxins.

Enzyme-linked immunosorbent assay (ELISA) is a popular antibody assay format and usually designed as a "sandwich" assay, where an antibody is used to capture the antigen and a second antibody conjugated with an enzyme is used for detection. The basic concept of ELISA has been adapted to various formats and even automated and it can be done in microtiter plate wells, dipsticks, paddles, membranes, pipet tips, etc., and using a variety of detection systems, including chromogenic and fluorogenic substrates and fluorescent or chemiluminescent labels.

Recently, immunoprecipitation or immunochromatography assays have become popular for detecting pathogens in foods. The assay is also a "sandwich" antibody test but, instead of conjugates, the detection antibody is labeled with colored latex beads or with colloidal gold to give a visible band of immunoprecipitation. Fashioned after home pregnancy tests, these assays use small, disposable plastic strips or dipsticks that require no washing or

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manipulations, so are extremely simple, and can yield results within minutes, post enrichment.

In food testing, another use for antibodies is for selective capture of bacteria (*16, 17*). In immunomagnetic separation (IMS), antibody bound to magnetic beads are used to selectively capture bacteria from enrichment media thereby, shortening culture enrichment time. IMS is analogous to selective enrichment, except that it can be done within an hour, so it is faster and does not use harsh chemicals or antibiotics that may cause cell stress or injury. Although IMS will not yield a pure culture, the target organism is greatly concentrated and can be further tested by plating, serological, genetic or other tests. Coupling IMS to other tests generally improves overall detection efficiency of assays.

Antibodies are also used extensively as the specificity component in next generation tests like biosensors that detect physicochemical changes in a matrix caused by antigen-antibody binding (*18, 19, 20*). Biosensors for detecting food borne pathogens are already commercially-available and although most still require a short enrichment step in the analysis of foods, biosensors may potentially enable in-line monitoring for pathogens and toxins during food processing (*21*). Antibody-based assays for detecting bacteria and toxins are shown in Tables II and III, respectively.

## Applications, Validation, and Impact of Rapid Methods

Most rapid methods are single target tests and continue to require some culture enrichment prior to testing. The benefits of enrichment however, outweigh the sacrifices in speed of analysis, as enrichment dilutes out effects of inhibitors, allows the repair of stress-injured cells, and also helps to differentiate viable from non-viable cells. But even with the enrichment steps, rapid methods are still faster, more sensitive, and more specific than conventional methods that are being used for the detection of pathogen and toxins in foods. As a result, they are well suited for screening large numbers of food samples for the presence or absence of a particular target. In use as a screening tool, negative results by rapid method are accepted but positive results are regarded only as presumptive and needs to be confirmed. Since confirmation is often done by conventional methods, it is time consuming and will extend analysis time by a few days. This however, may not be an imposing requirement as negative results are most often encountered in food testing.

Because rapid methods use various technologies, their detection sensitivities vary greatly (Table IV) and may be food dependant as some assays work better in some foods than others. It is therefore, critical that rapid methods are evaluated to ensure effective performance in specific foods. Comparatively, testing of rapid versus standard method, as done in validation studies, are also critical to determine false-positive or false-negative rates. Since negative results from rapid methods are accepted, false-negatives if not recognized, are

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## Table II. Partial Listing of Antibody-Based Rapid Methods

| *Bacteria* | *Assay* | *Format*[a] | *Maker* |
|---|---|---|---|
| *Campylobacter* | Campyslide | LA | Becton Dickinson |
| | Meritec | LA | Meridian |
| | Microscreen | LA | Microgen |
| | Dryspot | LA | Oxoid |
| | Pathatrix | IMS | Matrix Microscience |
| | Assurance Gold | ELISA | BioControl |
| | TransiaPlate | ELISA | Diffchamb AB |
| | Alert | ELISA | Enojen |
| | VIA | ELISA | TECRA |
| | EIAFoss | ELISA | FOSS |
| | VIDAS | ELFA | bioMerieux |
| | Singlepath | Ab-ppt | Merck |
| | PATHIGEN | ECL | BioVeris |
| | DIA/PRO | biosensor<br>UMEDIK | |
| | DETEX | ElectroIA | Molecular Circuitry |
| *Escherichia coli* O103 | SeroCheck | LA | Oxoid |
| | Dyanbeads | IMS | Dynal |
| O111 | O111-F | LA | Denka Seiken |
| | SeroCheck | LA | Oxoid |
| | Dyanbeads | IMS | Dynal |
| O128 | SeroCheck | LA | Oxoid |
| O145 | SeroCheck | LA | Oxoid |
| | Dyanbeads | IMS | Dynal |
| O26 | O26-F | LA | Denka Seiken |
| | SeroCheck | LA | Oxoid |
| | Dyanbeads | IMS | Dynal |
| O91 | SeroCheck | LA | Oxoid |
| H7 | RIM | LA | REMEL |
| | Wellcolex | LA | Murex |
| O157 | RIM | LA | REMEL |
| | Dryspot | LA | Oxoid |
| | Prolex | LA | Pro-Lab |
| | EcolexO157 | LA | Orion Diagnostica |
| | Wellcolex | LA | Murex |
| | O157-AD | LA | Denka Seiken |
| | Captivate | LA | IDG/LabM Ltd |
| | Microscreen | LA | Microgen |
| | ANI *E. coli* O157 | LA | ANI Biotech |
| | Pathatrix | IMS | Matrix Microscience |
| | Dyanbeads | IMS | Dynal |
| | VIP | Ab-ppt | BioControl |
| | Reveal | Ab-ppt | Neogen |
| | NOW | Ab-ppt | Binax |
| | QUIX | Ab-ppt | Univ. Health Watch |
| | ImmunoCardSTAT | Ab-ppt | Meridian Diag. |
| | PATH-STIK | Ab-ppt | Celsis |
| | TransiaCard | Ab-ppt | Diffchamb AB |
| | RapidChek | Ab-ppt | Strategic Diag. Inc |
| | Singlepath | Ab-ppt | Merck |

*Continued on next page.*

**Table II. *Continued.***

| *Bacteria* | *Assay* | *Format*[a] | *Maker* |
|---|---|---|---|
| | Eclipse O157:H7 | Ab-ppt | Eichrom Technologies |
| | O157 Antigen | Ab-ppt | Morningstar Diag. |
| | SMART-II | Ab-ppt | New Horizon |
| | O157 Coli-Strip | Ab-ppt | Coris BioConcept |
| | PetrifilmHEC | blotEIA | 3M |
| | EZcoli | tubeEIA | Difco |
| | Assurance | ELISA | BioControl |
| | HECO157 | ELISA | 3M Canada |
| | TECRA | ELISA | Tecra |
| | E. coli O157 | ELISA | LMD |
| | PremierO157 | ELISA | Meridian |
| | Transia Plate O157 | ELISA | Diffchamb AB |
| | Ridascreen | ELISA | rBiopharma |
| | Colortrix | ELISA | Matrix Microscience |
| | EIAFoss | ELISA | FOSS |
| | VIDAS | ELFA | bioMerieux |
| | VIDAS ICE | ELFA | bioMerieux |
| | PATHIGEN | ECL | BioVeris |
| | DETEX | electroIA | Molecular Circuitry |
| | DIA/PRO | biosensor | UMEDIK |
| | RBD3000 | biosensor | AATI |
| O157/O26 | EHEC-Tek | ELISA | Organon-Teknika |
| *Listeria* spp. | Microscreen | LA | Microgen |
| | ListerTest | IMS | VICAM |
| | Dyanbeads | IMS | Dynal |
| | Pathatrix | IMS | Matrix Microscience |
| | Singlepath | Ab-ppt | Merck |
| | VIP | Ab-ppt | BioControl |
| | Clearview | Ab-ppt | Unipath |
| | Reveal | Ab-ppt | Neogen |
| | Listeria-TEK | ELISA | Organon Teknika |
| | TECRA VIA | ELISA | TECRA |
| | Assurance | ELISA | BioControl |
| | Transia Plate | ELISA | Diffchamb AB |
| | VIDAS LIS | ELFA | bioMerieux |
| | EIAFoss | ELISA | FOSS |
| | UNIQUE | cap.EIA | TECRA |
| | PATHIGEN | ECL | BioVeris |
| | RBD3000 | biosensor | AATI |
| | DIA/PRO | biosensor | UMEDIK |
| | DETEX | ElectroIA | Molecular Circuitry |
| *L. monocytogenes* | VIDAS LMO | ELFA | bioMerieux |
| | TransiaPlate | ELISA | Diffchamb AB |
| *Salmonella* | Bactigen | LA | Wampole |
| | Spectate | LA | Rhone-Poulenc |
| | Microscreen | LA | Microgen |
| | Wellcolex | LA | Lab. Wellcome |
| | Serobact | LA | REMEL |
| | RapidTest | LA | Unipath |
| | ANI Salmonella | LA | ANI Biotech |
| | Salmonella Verify | LA | VICAM |
| | Salmonella Seiken | LA | Denka Seiken |

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**Table II.** ***Continued.***

| *Bacteria* | *Assay* | *Format*[a] | *Maker* |
|---|---|---|---|
| | Dynabeads | IMS | Dynal |
| | Salmonella Screen | IMS | VICAM |
| | PATHATRIX | IMS | Matrix Microscience |
| | Salmonella-TEK | ELISA | Organon Tek. |
| | TECRA VIA | ELISA | TECRA |
| | EQUATE | ELISA | Binax |
| | BacTrace | ELISA | KPL |
| | Assurance | ELISA | BioControl |
| | Salmonella | ELISA | GEM |
| | LOCATE | ELISA | Rhone-Poulenc |
| | Colortrix | ELISA | Matrix Microscience |
| | Salmonella | ELISA | Bioline/Mast Diag. |
| | Transia Plate Gold | ELISA | Diffchamb AB |
| | Salmotype | ELISA | Labor Diag. Leipzig |
| | EIAFoss | ELISA | FOSS |
| | VIDAS SLM | ELFA | bioMerieux |
| | VIDAS ICS | ELFA | bioMerieux |
| | PATHIGEN | ECL | BioVeris |
| | DIA/PRO | biosensor | UMEDIK |
| | RBD3000 | biosensor | AATI |
| | Salmonella 1-2 | Ab-diff. | BioControl |
| | CHECKPOINT | blot | KPL |
| | UNIQUE | cap.EIA | TECRA |
| | PATH-STIK | Ab-ppt | Celsis |
| | Reveal | Ab-ppt | Neogen |
| | Clearview | Ab-ppt | Unipath |
| | TransiaCard | Ab-ppt | Diffchamb AB |
| | Singlepath | Ab-ppt | Merck |
| | SMART-II | Ab-ppt | New Horizon |
| *S. enteritidis* | SE Verify | LA | VICAM |
| | Salmonella 1-2 SE | Ab-diff. | BioControl |
| | CHEKIT | ELISA | Bommeli Diag. |
| | FlockChek | ELISA | IDEXX |
| *Shigella* | Bactigen | LA | Wampole |
| | Wellcolex | LA | LabWellcome |
| *Staphylococcus Aureus* | Staphyloslide | LA | Becton Dick. |
| | AureusTest | LA | Trisum |
| | StaphyTest plus | LA | Oxoid |
| | Microscreen | LA | Microgen |
| | ANI S. aureus | LA | ANI Biotech |
| | TECRA | ELISA | TECRA |
| *Vibrio cholera* | V. cholera 01-AD | LA | Denka Seiken |
| *Yersinia enterocolitica* | ANI | LA | ANI Biotech |
| | Microscreen | LA | Microgen |

[a] LA: latex agglutination; IMS: immunomagnetic separation; ELISA: enzyme linked immunosorbent assay;ELFA: enzyme linked fluorescence assay; Ab-ppt: immuno-precipitation; ECL: electrochemiluminescence; ElectroIA : electroimmunoassay; blot EIA : blot enzyme immunoassay; cap.EIA : capture EIA; Ab-diff. : antibody diffusion.

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**Table III. Partial Listing of Rapid Methods for Bacterial Toxins**

| *Bacteria* | *Toxin* | *Assay* | *Format*[a] | *Company* |
|---|---|---|---|---|
| ***Bacillus cereus*** | diarrheal enterotoxin | BDEVIA | ELISA | TECRA |
| | | BCET | RPLA | Denka Seiken |
| ***Clostridum botulinum*** | A,B,E,F | ELCA | ELISA | Elcatech |
| | | Bot toxin | ELISA | METAbiologics |
| | | Smart-II | Ab-ppt | NewHorizon |
| | | BTA | Ab-ppt | Alexeter Tech |
| ***C. perfringens*** | enterotoxin | PET | RPLA | Denka Seiken |
| ***Escherichia coli*** | Shiga toxin | Verotest | ELISA | Microcarb |
| | | Premier | ELISA | Meridian |
| | | VTEC | RPLA | DenkaSeiken |
| | | Screen | LA | DenkaSeiken |
| | | TaqMan | rtPCR | Perkin Elmer |
| | | Ridascreen | ELISA | rBiopharma |
| | | Duopath | Ab-ppt | Merck KgaA |
| | | ProSpecT | ELISA | REMEL |
| | | Transiaplate | ELISA | Diffchamb AB |
| | Labiletoxin | VET | RPLA | DenkaSeiken |
| | Stabletoxin | COLIST | ELISA | DenkaSeiken |
| | | E. coli ST | ELISA | Oxoid |
| ***Staphylococcus aureus*** | enterotoxin | SET | RPLA | Denka Seiken |
| | | SETVIA | ELISA | TECRA |
| | | SETID | ELISA | TECRA |
| | | Transiatube | ELISA | Diffchamb AB |
| | | TransiaPlate | ELISA | Diffchamb AB |
| | | Ridascreen | ELISA | rBiopharma |
| | | VidasSET | ELFA | bioMerieux |
| | SEB | SMART | Ab-ppt | New Horizon |
| | | BTA | Ab-ppt | Alexeter Tech |
| ***Vibrio cholera*** | CT | VET | RPLA | Denka Seiken |
| ***V. parahaemolyticus*** | hemolysin | KAP | RPLA | DenkaSeiken |

[a] See previous tables.

**Table IV. Detection Sensitivities of Various Assays**

| *Assay* | *Bacteria (cells/g)* | *Toxins (ng/ml)* |
|---|---|---|
| Culture | $10^7$ - $10^8$ | NA[a] |
| Adenosine triphosphate (ATP) | $10^4$ | NA |
| Latex agglutination (LA) | $10^7$ | NA |
| Reverse Passive LA (RPLA) | NA | 0.5 - 4.0 |
| Enzyme linked immunosorbent assay | $10^4$ - $10^7$ | 0.01 - 1.0 |
| Immunomagnetic seperation (IMS) | $<10^3$ | NA |
| Immunodiffusion (1-2 Test) | $10^5$ - $10^6$ | 5 - 100 |
| Immunoprecipitation (Ab-ppt) | $10^4$ - $10^8$ | NA |
| DNA Probe | $10^4$ - $10^6$ | NA |
| phage (*lux* or *ina*) | $10^1$ - $10^2$ | NA |
| PCR | $10^1$ - $10^2$ | NA |
| Biosensor | $10^1$ - $10^2$ | NA |

[a]NA - information not available or applicable.

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precarious in terms of food safety, for if the product is consumed, it may cause human infections.

There are many internationally recognized method validation programs and many regulatory agencies also have internal validation procedures (*22*), but in the United States, methods most often become official or standard methods after been subjected to the collaborative study program of the Association of Official Analytical Chemists (AOAC) International (*23*). AOAC validation is an extensive, multi-lab, comparative testing of the new versus standard method using multiple samples and replicates of food types seeded with various levels of the target pathogen. Once the study data have been reviewed and approved, an official status is granted and the methods must be performed exactly as specified in the protocol.

The introduction of commercially-available rapid methods also had a great impact on validation programs. In response to increased demands for validation, AOAC Research Institute (RI), an affiliate of AOAC was formed in 1991, with the sole function of managing the Performance Tested Methods Program for methods that had proprietary technologies. The RI program is simpler and shorter but attained no official status (*23*).

More recently, validation programs evolved even further with the introduction of the AOAC eCAM system, which took into account the needs of the regulatory agencies. Prioritized into 5 categories based on the degree of validation (Reference/Regulatory [RRM]; Harmonized Collaboratively Validated [HCV]; Multiple Laboratory Validated [MLV]; Single Lab Validated [SLV]; and Developmental Non-Validated [DNV]), the eCam system is intended to enable the user to determine which extent of method validation is suitable for their purposes.

Aside from speed and simplicity, another benefit of rapid methods is better sensitivity. However, improvements in sensitivity can also create interesting problems in food testing, especially on the current specification of "zero tolerance" or "absence" established for pathogens and toxins in ready-to-eat foods. The determination of the "zero" criteria is method-dependant and comformity has long been monitored using conventional methods. The problem with increased assay sensitivity however, is that it may give rise to situations where foods previously analyzed by traditional methods and found to have no pathogens or toxins may no longer meet the same specifications if more sensitive methods are used. While this is beneficial to the consumer in terms of food safety, it may create interesting challenges to the quality control programs of the food industry and also to the regulatory positions of the state and federal agencies. Also, each time a more sensitive method is validated to become an official method, the "zero" criteria also become more stringent.

Rapid methods development continue to advance at a great pace and will have even more impact on future food diagnostic methods. Next generation tests already exist and are even faster, more sensitive and capable testing multiple targets simultaneously. But the problems of sampling, the complexity of foods, and the required sample enrichment or preparation procedures prior to testing, continue to challenge the development of rapid methods to test for pathogens and toxins in foods.

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## Chapter 3

# Nucleic Acid-Based Diagnostic Methods

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Assays based on the polymerase chain reaction (PCR) are now accepted methods for rapidly confirming the presence or absence of specific pathogens in foods and other types of samples. Conventional PCR requires the use of agarose gel electrophoresis to detect the PCR product; whereas, real-time PCR combines DNA amplification with fluorescent probe detection of the amplified target sequence in a closed tube format. Both conventional and real-time PCR and multiplex PCR assays have been developed for detection of *E. coli* O157:H7, *Campylobacter* species, simultaneous detection of *E. coil* O157:H7 and *Salmonella* species, and for specific detection of different *E. coli* serogroups based on unique gene sequences in the *E. coli* O antigen gene clusters. Microarrays, consisting of many probes complementary to pathogen-specific gene sequences bound to a solid substrate can hybridize multiple DNA targets simultaneously; therefore, microarrays have tremendous potential for detection, identification, and characterization of pathogens. Novel methods combining on-chip PCR of template DNA and simultaneous sequence-specific detection of amplification products on a solid phase show great potential for routine testing of bacterial pathogens in foods.

In Advances in Microbial Food Safety; Juneja, V., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

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Detection and identification of microorganisms in foods, animal feces, and environmental samples have historically been of limited diversity relying on cultural detection techniques, which are time consuming and labor intensive. A straight forward approach for conceptualizing detection technologies and their feasibility is to categorize them into three groups. Traditional cultural methods, regarded as the "gold standard", involve enrichment of the sample in liquid medium, plating onto selective agar/s, and confirmation of the pure culture isolate using a series of morphological, biochemical, serological, and other tests. Immunological-based assays rely on the binding of an antibody to an antigen of the bacterium, and genetic-based methods rely on binding of segments of nucleic acids to bacterial DNA targets. Genetic methods include the polymerase chain reaction (PCR), and DNA hybridization assays, including DNA microarray formats. The term "rapid method" appeared in the literature within the past 20 years, and refers to methods that expedite the detection process. Formats of rapid methods include commercially available miniaturized biochemical kits for identification of pure culture isolates, immunoassays including latex agglutination assays or enzyme-linked immunosorbent assays, and genetic-based assays such as the PCR.

## Polymerase Chain Reaction (PCR)

The PCR is a powerful technique that has transformed basic biology and has become a widely used tool for the diagnosis of microbial infections and genetic diseases, as well as for detection and identification of pathogens in food and environmental samples. Assays based on the PCR are now accepted methods for rapidly confirming the presence or absence of specific pathogens in foods. The choice of genomic or plasmid DNA region/s selected for amplification determines the specificity of the assay for the target pathogen/s. Target sequences include the rRNA operon, virulence genes, or other unique DNA regions or genes. Conventional PCR methods for pathogen detection generally involve four steps: (1) nucleic acid extraction; (2) DNA amplification; (3) product detection by agarose gel electrophoresis; and (4) amplicon confirmation. The PCR product/s is/are visualized and sized by performing agarose gel electrophoresis and staining with ethidium bromide. To confirm that the amplicon is the correct PCR product, Southern blot analysis or enzyme-linked hybridization capture assays can be performed. Combining the PCR with a hybridization step enhances assay sensitivity and specificity.

With multiplex PCR, more than one target DNA sequence can be amplified and detected in a single reaction. For a successful multiplex PCR assay, however, it is important to optimize reaction parameters, including the relative concentration of reaction components and the cycling temperatures, to avoid the formation of spurious amplification products and uneven amplification of target sequences. Multiplex PCR can result in considerable savings in time, effort, and cost in the laboratory. We have developed multiplex PCR assays for detection of *E. coli* O157:H7 targeting up to five sequences, *fliC*$_{h7}$, *stx*$_1$, *stx*$_2$, *eaeA*, and *hly*$_{933}$ in one reaction (*1*). The multiplex PCR reduces the time required for detection and for confirmation of *E. coli* O157:H7 isolates since H (flagellar antigen type) typing and determination of virulence gene profile can be accomplished in a single rapid assay. Furthermore, a multiplex PCR assay was developed to detect *E. coli* O157:H7 (*eae*, conserved sequences of *stx*$_1$ and *stx*$_2$, and *hlyA*$_{933}$ genes) and *Salmonella* spp. (*invA* gene) simultaneously in ground beef, apple cider, bovine feces, and beef carcass wash water (Figure 1) (*2*).

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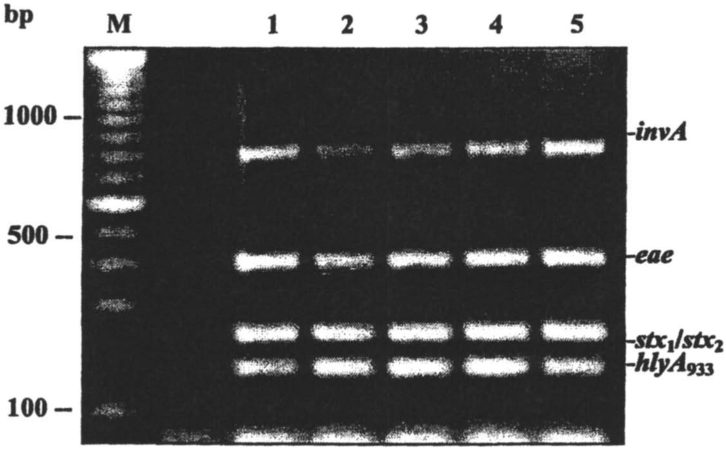

*Figure 1. Ethidium bromide-stained agarose gel showing PCR products obtained following immunomagnetic separation to capture the target bacteria and multiplex PCR of E. coli O157:H7 and S. Typhimurium DNA from artificially-inoculated bovine feces after 20 h of enrichment in buffered peptone water containing 0.02 mg/ml of novobiocin.*

A number of other nucleic acid amplification techniques have been described, including isothermal amplification methods known as nucleic acid sequence-based amplification (NASBA), and strand displacement amplification (SDA) (*3*). The NASBA method uses three enzymes - a reverse transcriptase, RNaseH, and T7 polymerase - which act in concert to amplify bacterial RNA (*4*). This method has been employed for detection of a number of pathogens including *Campylobacter* spp., *L. monocytogenes*, *Salmonella* spp., *Cryptosporidium parvum*, and food-borne viruses.

Routine use of the PCR in food testing has been hindered by the relative complexity of the assay and the need for clean environments to avoid carryover of PCR products from one reaction to the next resulting in false positive results. However, recent developments in PCR methodology have resulted in more user-friendly procedures that can be performed routinely by users with minimal skills. PCR test kits targeting specific food-borne pathogens are commercially-available. These include the BAX system kits (Qualicon, Wilmington, DE), which involve the use PCR tablets containing all of the reagents needed to perform the PCR, the TaqMan system from Applied Biosystems (Foster City, CA), and the Probelia system from Sanofi Diagnostics (Pasteur, France, but now merged with Bio-Rad Laboratories, Richmond, CA).

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## Real-Time PCR

An important advance in recent years is the development of homogenous assays permitting real-time detection of target nucleic acid in a closed system, in which the PCR and amplicon detection are performed in the same reaction vessel. Real-time PCR has many applications including gene expression analysis (*5*), single nucleotide polymorphism (SNP) typing (*6*), and pathogen detection (*7, 8, 9, 10*). Although real-time PCR is not currently used widely for routine pathogen testing by the food industry, an increasing demand for high-throughput screening in the clinical and pharmaceutical industries has produced several technological developments in methods for detecting and analyzing biological molecules, many of which could be applied to problems in the food industry. Compared to conventional PCR, real-time PCR methods offer a number of advantages. Real-time PCR assays are performed using closed systems; therefore, there is lower potential for cross contamination and false positive results. Secondly, there is a shorter analytical turnaround time and higher sensitivity and precision with real-time PCR compared to conventional PCR. The PCR products are detected as they accumulate during a real-time PCR reaction; therefore, there is no need for post-PCR processing steps. There is a larger quantitation range for real-time PCR compared to traditional PCR (5-6 logs versus 2-3 logs, respectively), and there are greater assay capabilities for real-time PCR instruments, including quantitative, qualitative, mutation, and multiplex assays.

Real-time PCR systems rely upon detection and quantitation of signal generated from a fluorescent reporter. The signal produced by the reporter increases in proportion to the amount of PCR product produced. The product yield (fluorescence) is plotted against cycle number yielding a curve that represents the accumulation of PCR product over the duration of the PCR reaction. The log-linear phase of the reaction is used to determine the cycle threshold ($C_t$) for each sample. The $C_t$ is defined as the first cycle in which there is a significant increase in fluorescence above a specified threshold. For quantitating the PCR product, a standard curve is generated using $C_t$ values for a series of reactions containing a known quantity of target DNA. Quantification is performed by comparing the $C_t$ values of unknown samples against the standard curve or against the $C_t$ values of an internal standard.

There are different fluorescence systems that can be employed for detection of production of the PCR product, and among the various chemistries available, SYBR Green I is the most economical and convenient to use. SYBR Green I is a thermostable intercalating dye that binds double-stranded DNA resulting in an increase in fluorescence as the amount of PCR product increases. In assays using SYBR Green I, products are detected by programming the real-time PCR instrument to perform a melt curve at the end of the reaction. A drop in fluorescence is observed at the point in which the PCR product melts due to dissociation of the dye from the double-stranded DNA. The specific PCR product has a unique $T_m$, thus melt curves can distinguish between specific and non-specific products, including primer dimers.

Dual-labeled (TaqMan) probes or molecular beacons are oligonucleotides that contain fluorescent and quenching dyes at the 5' and 3' ends, respectively. TaqMan probes bind to an internal region of the PCR product. During replication of the template, the polymerase exonuclease activity causes cleavage of the probe separating the reporter and quenching dyes, resulting in a measurable increase in fluorescence intensity. Real-time PCR assays based on the use of TaqMan probes for detection of food-borne pathogens have been described *(8, 10)*. Molecular beacons are oligonucleotides with a hairpin structure consisting of a sequence-specific portion (loop) and complementary arm sequences located on either side of the probe sequence. The complementary arm sequences that form the stem of the hairpin are end-labeled with the fluorophore and the quencher dyes. During the reaction, the probe sequence in the loop hybridizes to a complementary sequence within the PCR product. The conformational change that occurs distances the quencher from the reporter dye, yielding fluorescence (*9, 11, 12*). The fluorescence resonance energy transfer (FRET) principle makes use of two oligonucleotide probes, one labeled with a donor fluorochrome at the 3' end and the other labeled with an acceptor dye at the 5' end. The probes hybridize to the target sequences so that they are distanced by one or a few bases and are oriented head-to-tail. When in that position, the energy emitted from the donor excites the acceptor dye, which then emits fluorescent light at a longer wavelength. The amount of target DNA produced is proportional to the ratio of the fluorescence of the donor and the acceptor. Fluorescence is measured during the annealing step of the PCR when

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both probes hybridize to the target DNA (*13*). Other types of fluorescence systems developed for real-time PCR assays include Scorpion probes, LUX (Light Upon Extension) primers that are designed to be self quenched until they are incorporated into the PCR product resulting in an increase in fluorescence due to a change in the secondary structure, Amplifluors, MGB Eclipse probes (*14*), and others.

Several instruments currently available for performing real-time PCR include the LightCycler (Roche Diagnostics Corp.), the RAPID (Idaho Technologies), the iCycler iQ (Bio-Rad), the MX4000 (Stratagene), the Rotor Gene (Corbett Research), the ABI Prism 7000 and 7900HT (Applied Biosystems), the DNA Engine Opticon (MJ Research), and the Smart Cycler (Cepheid, Inc.). The RAPID and Smart Cycler instruments, originally designed in conjunction with the military to detect biological warfare agents in the field, are available as portable instruments. The design of the Smart Cycler, which was first described by Northrup (*15*), is unique compared to other real-time PCR platforms because each processing block contains 16 independently controlled, programmable I-CORE (Intelligent Cooling/heating Optical Reaction) modules. Sixteen different PCR protocols can be run simultaneously, which facilitates optimization of PCR assays. Up to 6 Smart Cycler processing blocks can be linked together allowing simultaneous analysis of 96 discrete samples. The LightCycler and the instruments from Applied Biosystems can been coupled with automated nucleic acid extraction instruments called the MagNa Pure LC and ABI Prism 6700 or 6100, respectively.

Real-time reverse transcriptase PCR (RT-PCR) using viral RNA or bacterial mRNA instead of DNA can also be performed. Reverse transcriptase is used to amplify RNA into cDNA. This is followed by real-time PCR, which copies the cDNA while incorporating fluorescent dyes or probes into the product. Fabre et al. (*16*) used a one-step real-time RT-PCR assay employing TaqMan probes for detection and quantitation of the Barley yellow dwarf virus. The assay was 10 to 1000 times more sensitive than standard RT-PCR and ELISA assays.

In multiplex real-time PCR assays, multiple sequences are amplified simultaneously in a single reaction, using probes labeled with differently colored fluorophores that have unique emission spectra. A multiplex PCR using TaqMan probes was developed to detect *Ralstonia solanacearum*. (*17*). One probe labeled with the FAM dye was used to detect all biovars of the organism, while the other probe labeled with the VIC dye detected only biovar 2A. A third primer set and probe set targeting the potato cytochrome oxidase gene was used as an internal control for the real-time PCR assay. Bellin et al. (*18*) developed a multiplex PCR targeting the genes encoding Shiga toxin 1 and Shiga toxin 2 in Shiga toxin-producing *E. coli* using FRET hybridization probes and the LightCycler instrument.

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Real-time PCR assays have been employed for detection and quantification of food-borne pathogens found in various types of samples (*19*, *20*, *21*). The power of real-time analysis is expanded by the ability to multiplex probes with different dyes, thus fewer reactions are needed to test a sample. Multiplex PCR assays for detection and differentiation of food-borne pathogens have been described (*22*, *23*). A multiplex real-time PCR assay employing TaqMan probes was developed to detect *E. coli* O157:H7 in foods. Four target sequences of the *E. coli* O157:H7, $fliC_{h7}$, $rfbE_{O157:H7}$, $stx_1$, and $stx_2$ genes were amplified simultaneously. The probe for the $fliC_{h7}$ PCR product was labeled with 6-carboxy-4,7,2',7'-tetrachlorofluorescein (TET) and the Black Hole Quencher 1 dye, the probe for $rfbE_{O157:H7}$ with 6-carboxyfluorescein (FAM) and Black Hole Quencher 1, and the probes for $stx_1$ and $stx_2$ were both labeled with Texas Red and Black Hole Quencher 2. Ground beef samples (25 g) were inoculated with ca.1 to 5 CFU of a cocktail of three strains of *E. coli* O157:H7, stored at 4°C for 72 h or at -20°C for 2 weeks, then subjected to enrichment in 225 ml of Rapid-Chek *E. coli* O157:H7 enrichment medium, BCM O157:H7 (+) broth, and modified *E. coli* broth containing novobiocin for 8 and 20 h at 42°C at 150 rpm. DNA extraction using the PrepMan Ultra (Applied Biosystems) reagent was performed using 1 ml of the enrichments. *E. coli* O157:H7 was detected in enrichments incubated for 8 h by the real-time multiplex PCR assay using the Smart Cycler. Thus, the assay can be employed for rapid detection of *E. coli* O157:H7 in ground beef, and potentially other types of samples as well.

## PCR-Based Typing of *E. coli* strains

Serotyping of *Escherichia coli* strains involves performing agglutination reactions using antisera raised against the ca. 180 O and 56 H serogroups antigens. The method is labor intensive, and several days are required to obtain results. Characteristically, genes specific to O antigen synthesis are located in the O antigen gene cluster (*rfb*) between the *galF* and *gnd* genes on the *E. coli* chromosome. Knowledge of the DNA sequence of genes within each of the clusters allows identification of unique sequences that can be used for the design of serogroup-specific PCR assays. We have determined the sequence of several *E. coli* O antigen gene clusters, including *E. coli* O26, O103, O104, O113, O121, O145, and O157 and developed serogroups-specific PCR assays targeting the *wzx* (O antigen flippase) and/or *wzy* (O antigen polymerase) genes of the respective O antigen gene clusters (Table 1) (*22*, *24*, *25*, *26*). The studies have shown that PCR assays targeting the *wzx* and *wzy* genes in the *E. coli* O antigen gene clusters are serogroups specific.

Determination of the presence of serogroup-specific genes, as well as the presence of the Shiga toxin gene/s and/or other virulence genes is important to

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determine if the *E. coli* strain is a potential human pathogen. Multiplex PCR assays were performed targeting both the *wzx* and *wzy* genes in a single PCR assay and targeting the Shiga toxin 1 (*stx*$_1$), Shiga toxin 2 (*stx*$_2$), *wzx*, and *wzy* genes in a single assay. Using DNA from two *E. coli* O103 clinical isolates in a multiplex PCR assay targeting *wzx*, *wzy*, *stx*$_1$, and *stx*$_2$, oducts for *wzx*, *wzy*, and *stx*$_1$ (321-, 280-, and 199-bp, respectively) were obtained for both strains (Figure 2). Thus, the two clinical *E. coli* O103 isolates possessed *stx*$_1$ but not *stx*$_2$. Using SYBR Green I in real-time PCR assays targeting the *E. coli* O103 *wzx* and *wzy* genes, the melting temperatures ($T_m$) for the *wzx* PCR product was 83.9 ± 0.3°C and for the *wzy* product was 80.3 ± 0.2°C (Figure 3). Therefore, the real-time PCR assays can be used to detect and easily distinguish the two products.

**Table 1. Oligonucleotide Primers used for Genetic-Based PCR Typing of *E. coli* Targeting the *wzx* and *wzy* Genes in the O Antigen Gene Clusters**

| *Gene target* | *Sequence of forward and reverse primers (5' to 3')* | *Amplicon size (bp)* |
|---|---|---|
| *wzx E. coli* O103 | TTGGAGCGTTAACTGGACCT<br>GCTCCCGAGCACGTATAAAG | 321 |
| *wzy, E. coli* O103 | ATACAAATGGCGTGGATTGG<br>GCCAGTAATTGACGTAACTGCTCT | 280 |
| *wzx, E. coli* O104 | ATTCATGACGCTAGAAC<br>TCACATGCACCAGTTAAG | 532 |
| *wzy, E. coli* O104 | AGTTCATTAGATCGAGGTT<br>CTCCTTGCAAATGTGCAA | 460 |
| *wzx, E. coli* O121 | TGGCTAGTGGCATTCTGATG<br>TGATACTTTAGCCGCCCTTG | 322 |
| *wzy, E. coli* O121 | GCAATGAGGACCGGTATATCTC<br>CACGCCCGTGTTAATATTCC | 318 |
| *wzx, E. coli* O145 | ACTGGGATTGGACGTGGATA<br>AGGCAAGCTTTGGAAATGAA | 222 |
| *wzy, E. coli* O145 | CTGTTGCTTCAGCCCTTTTC<br>GCAGCCCAATATGAAACCAT | 217 |
| *wzy, E. coli* O157 | CCTGTCAAAGGATAACCGTAATCC<br>TTTGTTCTCCGTCTTGTCCTAAACT | 112 |

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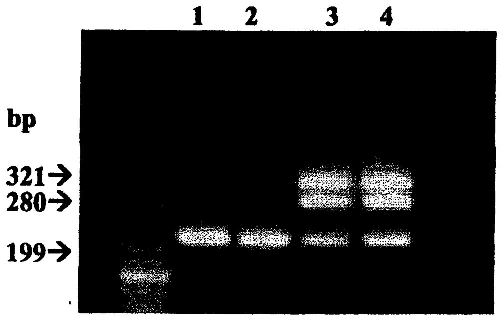

*Figure 2. Agarose gel electrophoresis showing multiplex PCR results using DNA from clinical isolates, E. coli O103:H25 (lane 1) and E .coli O103:H2 (lane 2), using primers targeting the* $stx_1$ *and* $stx_2$ *genes and DNA from E. coli O103:H25 (lane 3) and E. coli O103:H2 (lane 4) using primers targeting the* $stx_1$ *and* $stx_2$ *genes and the E. coli O103 wzx and wzy genes.*

## Microarrays for Pathogen Detection

The use of DNA microarrays, which consist of arrays of discretely located oligonucleotides or PCR products (the probe) attached to a solid support (usually glass) by a linker molecule, represents the latest development in pathogen detection technology. The recognition is based on hybridization of complementary strands between the probe and the target DNA molecule with fluorescence or chemiluminescence detection. Target DNA includes PCR products, genomic DNA, total RNA, cDNA, or plasmid DNA that incorporate a fluorescent label or compounds such as biotin permitting detection using conjugates of streptavidin. Following the hybridization and washing steps, the arrays are examined using a high-resolution scanner. Microarrays can be used to detect PCR products by hybridization of the amplicons to an array composed of pathogen-specific probes. Sergeev et al. (*27*) used degenerate primers to amplify as many as nine *Staphylococcus aureus* enterotoxin genes, followed by hybridization of the PCR products to microarrays containing oligonculeotide probes specific for each enterotoxin. Using immunomagnetic capture, PCR, and a microarray containing probes complementary to four virulence genes of *E. coli* O157:H7, Call et al. (*28*) were able to detect 55 CFU of *E. coli* O157:H7 per milliliter of chicken rinsate without an enrichment step. Detection of PCR products using the array was 32-fold more sensitive than gel electrophoresis. Chizhikov et al. (*29*) detected microbial virulence factors of multiple pathogens by multiplex PCR followed by hybridization of the PCR products to gene-

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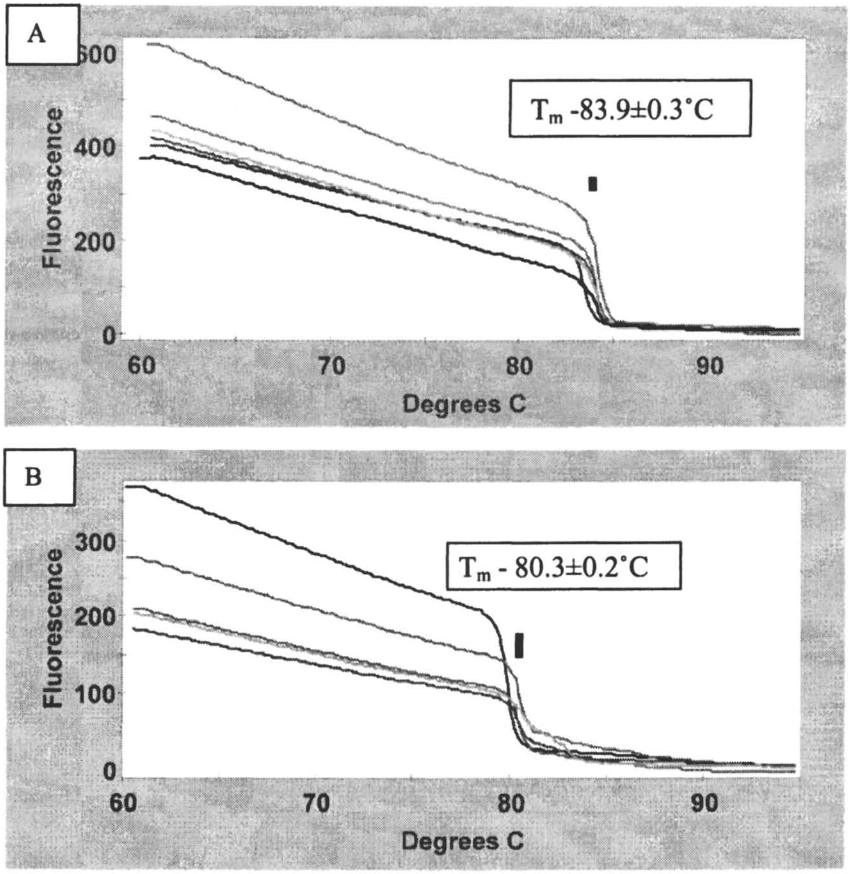

*Figure 3. Melting curve analysis following real-time PCR using the SYBR Green I dye and targeting the E. coli O103 wzx (A) and wzy (B) genes using 4 different DNA concentrations.*

specific oligonucleotides on microarrays. A comparison of DNA amplification strategies, including random primed amplification, isothermal Klenow fragment-based amplification, Φ29 DNA polymerase-based amplification, and multiplex PCR, were evaluated by subsequently hybridizing the amplicons to microarrays containing probes for *E. coli* O157:H7 (*30*). The study demonstrated that use of random amplification methods combined with detection using 70-mer oligonucleotide microarrays permitted amplification of the total DNA in a sample, while retaining specificity through hybridization to probes on the microarrays. Additionally, use of multiple probes ensures accurate identification of target pathogens. Microarrays can also be used for direct detection of DNA or RNA without the use of the PCR and can be used for genotyping or "fingerprinting" bacterial isolates. A recent review by Call et al. (*31*) describes a number of applications of microarray technology for pathogen detection and characterization.

Nucleic acid-based assays, such as the PCR, have shown promise as potential alternatives to culture techniques and immunoassays for detection of food-borne pathogens due to their increased rapidity, sensitivity, and specificity. Developments in PCR technology have resulted in rapid-cycle real-time assays that allow the user to monitor the accumulation of double-stranded DNA products during the PCR. Performing the PCR and detecting the PCR products all on a chip will soon become a reality. Microchip devices are under developments that integrate cell lysis, multiplex PCR amplification, electrophoretic separation of PCR products, and detection (*32*, *33*). Another new approach for detection, identification, and characterization of a target pathogen or potentially of multiple bacterial species simultaneously takes advantage of microarray technology.

Challenging problems that still remain in food testing include the development of food sampling and processing methods that ensure capture of low levels of target pathogens, separation from the food matrix, and concentration prior to detection using nucleic acid-based methods or other types of assays. Further research is needed to develop methods that combine different rapid methods for pathogen detection, including immunological and nucleic acid-based techniques. For example, antibodies can be used for capture and concentration of target organisms followed by nucleic acid-based methods for detection. Before the use of real-time PCR assays and microarrays for routine screening of samples for the presence of pathogenic organisms becomes a reality, additional research in the development of rapid, simple, and inexpensive assay systems for high-throughput automated sample processing and detection of pathogens in foods and other types of samples is critical.

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## Chapter 4

# Molecular Approaches to Immunological Biosensors: Phage Displayed Antibodies for the Detection of Foodborne Pathogenic Bacteria

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Traditional methods for the detection of microbial food-borne pathogens require 3-7 days to obtain a result. The introduction of HACCP plans and the desire to hold raw meat products in inventory until test results are confirmed have increased the need for more rapid detection methods. Recently, a variety of rapid methods (8-48 hours) have been developed. Many of these rapid methods utilize antibody molecules to capture and detect food-borne pathogens. Thus, the development of these immunosensor methods depends on the availability of antibodies with sufficient specificity. Rapid detection of *Listeria monocytogenes* has been hampered by the lack of polyclonal serum or monoclonal antibodies that can specifically detect the organism at the species level. Recently, antibody phage display has been employed to isolate a single-chain antibody fragment specific for *L. monocytogenes* and this single-chain antibody is being used to develop immunosensors for the detection of *L. monocytogenes* in food. The techniques should be applicable to other foodborne and emerging pathogens.

U.S. government work. Published 2006 American Chemical Society.

In Advances in Microbial Food Safety; Juneja, V., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

## Introduction

Incidental contamination of foods by pathogenic bacteria and/or their toxins is a serious threat to public health and the economy and intentional contamination of the food supply (bioterrorism) may pose an even greater threat. Each year in the United States foodborne diseases cause an estimated 76 million illnesses, 235,000 hospitalizations and 5000 deaths (*1*) with associated costs estimated to be as much as 6.7 billion dollars (*2*). Thus, detection of pathogenic bacteria in foods prior to distribution is critical for consumer protection and consumer confidence. The introduction of Hazard Analysis and Critical Control Point (HACCP) programs in the food industry , increased government oversight, and the desire to acquire results of pathogen testing prior to distribution (to prevent costly food recalls) have increased the demand for simple and cost-effective methods to rapidly detect pathogens and toxins at various stages of food production, processing and distribution. As a result, the 2005 market for testing food-pathogens is expected to grow to 34 million tests at a cost of $192 million (*3*).

Traditional microbiological methods for the detection of foodborne pathogens, while robust, are slow (3-7 days) and labor intensive, and more rapid alternatives are needed. Advances in biotechnology have lead to the development of a variety of more rapid (8-48 hours) biosensor-based methods for pathogen/toxin detection. Biosensors fall into two broad groups based upon the biological recognition components required for specificity: nucleic acid-based biosensors and immunologically-based biosensors. Immunologically-based biosensors rely on antibodies for assay specificity. Antibody phage display, a molecular method for the selection of antibodies with the desired affinity and specificity, is beginning to be applied in the field of food pathogen detection.

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## Methods for the Detection of Foodborne Pathogens

Methods for the detection of pathogens in food need to meet a number of criteria. They must be very sensitive since an infectious dose may be as low as a single pathogenic organism. They must be selective because pathogenic organisms are most often present as a very small fraction of a harmless microbial flora present in food. Speed is desired since modern food production and distribution systems operate rapidly. Current methods can be divided into two groups, conventional microbiological methods and rapid biosensor methods.

## Conventional Microbiological Methods

Conventional methods for the detection of foodborne pathogens utilize well established microbiological techniques. Food samples are subjected to a series of culture-based pre-enrichment and selective enrichment to increase the number of pathogens while minimizing the growth of the benign microbial flora. After enrichment, samples are grown on selective plating media in order to isolate individual bacterial colonies. Additional microbial, biochemical, or immunological characterization of individual bacterial clones is usually necessary to identify the pathogen. While these conventional methods can be both sensitive and selective, they are expensive, labor intensive, and slow, often taking 3 to 14 days to complete.

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## Rapid Biosensor Methods

At the core of all biosensor methods is a biorecognition component that imparts specific recognition of the target analyte (e.g., pathogenic bacterium). Biosensor-based methods take advantage of advances in biotechnology that have increased our understanding of biomolecular interactions to allow rational design and selection of biorecognition molecules and advances in electronics and microfabrication technologies that allow the detection of these intermolecular interactions. The basic components of a biosensor are represented schematically in Figure 1. Interaction between the target analyte and the biorecognition component of the biosensor results in the transduction of a signal which can be interpreted by a detector.

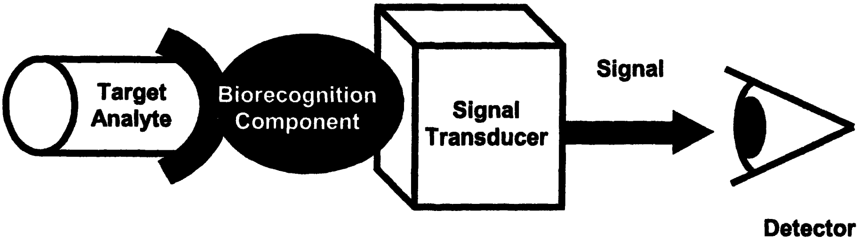

*Figure 1. Core components of a biosensor.*

Extensive reviews of the application of biosensors for the detection of foodborne pathogens have been published (*4-7*). A brief introduction to the topic is presented here.

The biorecognition component necessary for specific recognition of targets for biosensor-based processes divide them into two broad classes: nucleic acid-based biosensors and immunologically-based biosensors.

### *Nucleic Acid-Based Methods*

Nucleic acid-based biosensors take advantage of the extraordinary discriminatory power imparted by specific nucleotide sequences. These methods can be generally categorized as amplification methods, hybridization methods, or a combination of these two methods. Amplification methods involve the use of the polymerase chain reaction (PCR). PCR requires the use of a thermostable DNA polymerase and oligonucleotide primer sets to allow the amplification of specific DNA fragments. For PCR-based detection methods, the amplified DNA sequences are specific to the pathogen in question and the diagnostic DNA fragment is only amplified if the pathogen is present in the sample. For multiplex PCR, primer sets for the amplification of several target genes are included in a single reaction. The multiple primer sets may amplify more than one DNA fragment from a single pathogen to allow more confidence in the identification of the organism or may permit the amplification of DNA fragments from different pathogens to allow simultaneous detection of multiple pathogens from a single sample. The incorporation of fluorescent intercalating dyes in the PCR and the development of thermocyclers with fluorescence detection capabilities have permitted the interrogation of the sample between replication cycles. This technique is called real-time PCR (RT-PCR) because the DNA amplification is measured between cycles in "real-time" or quantitative PCR because it allows a quantitative determination of the target gene(s).

Nucleic acid hybridization methods take advantage of the base pairing between complementary DNA strands. Typically a specific single-stranded DNA fragment(s) is bound to a surface and presence of the pathogen is indicated by the hybridization of sample derived DNA fragments with the immobilized single-stranded DNA. Hybridization is typically detected via labeling the sample DNA or via changes in the surface properties (e.g., mass or refractive index) caused by the annealing of the sample DNA.

Two recently developed methods for nucleic acid-based detection of pathogens utilize a combination of DNA amplification and hybridization. One method is a variation of RT-PCR in which a self-quenching fluorescent oligonucleotide probe, called a molecular beacon, is included in the PCR. Between replication cycles conditions are created favoring the hybridization of the molecular beacon probe with the target PCR product. Hybridization of the molecular beacon with the PCR product eliminates the self-quenching properties of the molecular beacon resulting in fluorescent signal. Similar to RT-PCR using intercolating fluorescent dyes, molecular beacon PCR can be used for quantitation of the target DNA in the original sample. In addition, molecular beacon PCR can be multiplexed by incorporating different fluorescent dyes on each of the probes. A second PCR-hybridization technique for the detection and identification of foodborne pathogens is DNA microarrays. Although microarray

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technology has been most widely used for the examination of differences in gene expression under different environmental conditions or for comparison of whole genomes for phylogenetic/evolutionary strain comparisons, the method is beginning to be applied for the pathogen detection (*8-13*). Microarray technology involves spotting arrays of thousands of specific oligonucleotides or single stranded DNA fragments on the surface of a glass slide. Sample DNA is labeled, often via PCR, and the labeled DNA is hybridized to the array. The hybridization of the labeled sample DNA with the spots on the array is examined to determine the presences of specific genes (i.e., pathogens) in the sample. Because of the number of possible spots in a single array, microarray analysis offers tremendous potential of multiplexing for the detection of several genes and several pathogens in a single experiment.

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### *Immunologically-Based Methods*

The specific biorecognition component used in immunologically-based biosensors is the antibody (Figure 2). Immunological detection using antibodies is the only technique which has been used to successfully detect bacterial cells, spores, viruses, protein toxins, and small molecules. Any compound that can elicit an immune response can be used to generate antibodies for potential use as immunoreagents for detection.

Either polyclonal or monoclonal antibody preparations can be used as detection reagents. Polyclonal antibodies are the population of antibodies present in the serum of an immunized animal. This is a mixture of antibodies directed against the immunizing agent as well all other antigens to which the animal was previously exposed. For this reason, polyclonal antibody preparations may suffer from a lack of specificity against the immunizing agent. In addition, the volume of a polyclonal antibody preparation is limited to the volume of serum that can be collected from the immunized animal which can lead to batch to batch variation. Monoclonal antibody preparations overcome these limitations because they are prepared from a clone of an individual antibody producing B-cell made immortal by fusion to a tumor cell. These individual "hybridoma" cells secrete a single type of antibody. Individual cell lines can be used to prepare monoclonal antibody with well defined characteristics.

The typical antibody used in immunobiosensors is an immunoglobulin G which is composed of two heavy chains, each composed of three constant regions ($C_{H1}$, $C_{H2}$, and $C_{H3}$) and a single variable domain ($V_H$), and two light chains, each composed of a constant ($C_L$) and a variable ($V_L$) domain. The heavy and light chains combine in such a way that each antibody contains two antigen binding ($F_V$) regions. The Fv region is formed through the interaction of the $V_H$ and $V_L$ domains represented in Figure 2 by the region within the box. It is the specific and high affinity binding of antibody to antigen, conferred by this Fv region that makes antibodies so efficacious for biosensor development.

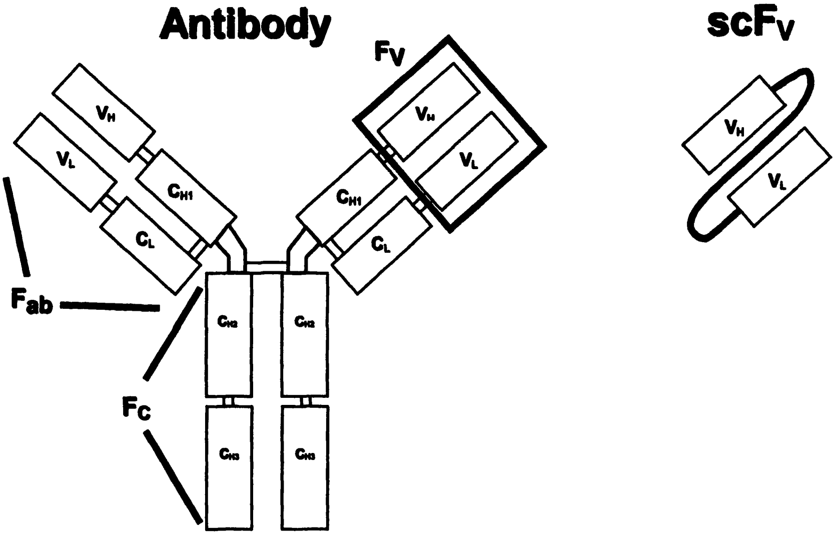

*Figure 2. Structure of antibody (IgG) and single-chain antibody (scFv).*

In immunologically-based biosensors, antibodies function as either capture or detection reagents. To perform as capture reagents, antibodies must first be bound to a solid surface. The solid support may take a variety of forms such as microtiter plates, glass slides, "dip-sticks", polystyrene beads, immunomagnetic beads, etc. The binding of the antibody to a solid support may be passive (such as binding to a polystyrene plate) or active (through a covalent linkage). One of the most common methods for immobilizing antibodies is through the interaction of biotinylated antibodies with a streptavidin coated surface. A variety of reporter molecules have been conjugated to antibodies to allow them to function as detection reagents, including chromogenic compounds, fluorescent compounds, fluorescent proteins, and enzymes. The type of reporter molecules employed dictates or is dictated by the type detector used in the biosensor.

Immunologically-based detection methods can be categorized as either direct immunoassays or indirect immunoassays (Figure 3). For biosensors employing a direct immunoassay, the detector measures a change in the capture surface upon binding of the antigen to the capture antibody. This may be a change in mass (piezoelectric sensors, quartz crystal microbalance or microcantilever), optical properties (surface plasmon resonance, total internal

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reflection fluorescence), or electrical properties (impedance/conductance) at the capture surface. For indirect immunoassays the pathogen is first captured and a detectable signal is generated only after the addition of a secondary detection antibody.

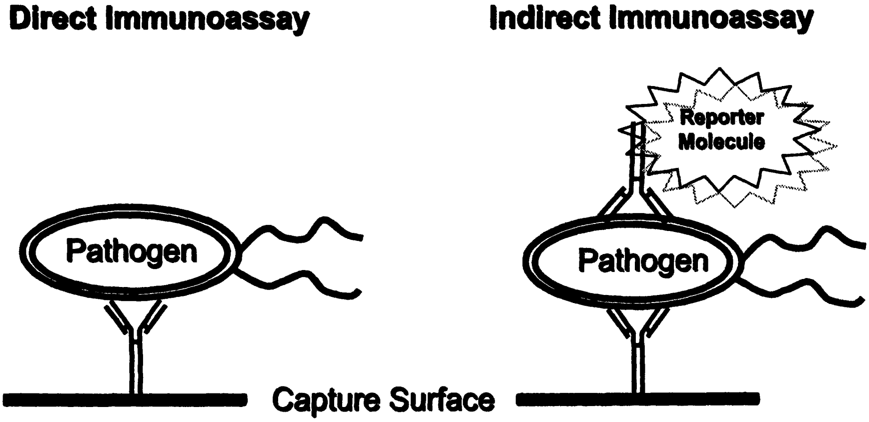

*Figure 3. Direct and indirect immunobiosensor formats. For the direct assay interaction of the pathogen is measured directly by a change at the capture surface. For indirect immunobiosensors a secondary labeled antibody is required to generate a detectable signal.*

The development of immunobiosensors is absolutely dependent upon the availability of antibodies of sufficient affinity and specificity to act as capture and detection reagents. Traditional methods for production of polyclonal and monoclonal antibodies have been useful for the production of immunoreagents for the capture and detection of a number of foodborne pathogenic bacteria. Nevertheless, traditional methods for antibody production are slow, expensive and highly empirical. Furthermore, these methods have not yielded antibodies with sufficient affinity and/or specificity for the detection of some foodborne pathogens. New molecular methods for the selection of antibodies hold promise for development of immunoreagents for these difficult to detect foodborne pathogens.

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## Antibody Phage Display

Recently, a molecular method for the selection of antibody fragments of desired specificity was developed (*14, 15*). This technique, antibody phage display, involves the cloning of nucleotide sequences encoding the antibody variable regions and display of the antibody fragments as a fusion protein on the surface of bacteriophage. The variable heavy and variable light sequences are linked via a flexible linker to generate a "single-chain antibody" (scFv) that retains the structural and functional properties necessary for antigen binding (Figure 2).

Antibodies of desired specificity are selected from a large collection of bacteriophage expressing unique antibody fragments through the binding of the antibody with the target of interest. Selection of antibodies from phage display libraries offers several advantages over traditional methods for antibody production (*16*): (1) *In vitro* selection of antibodies from phage display libraries allows greater flexibility in developing rational approaches to antibody selection; (2) The gene encoding the antibody fragment is isolated with the phage antibody; thus, capture and detection immunoreagents can be engineered via fusions to reporter genes; (3) Once a specific antibody fragment is identified, immunoreagent production is quicker and cheaper; (4) Smaller antibody fragments are less-prone to non-specific interactions; and (5) No animals need to be sacrificed. Phage display has been used to isolate antibody fragments that bind to bacterial pathogens (*17-20*) bacterial spores (*21, 22*), and bacterial protein toxins (*23-26*).

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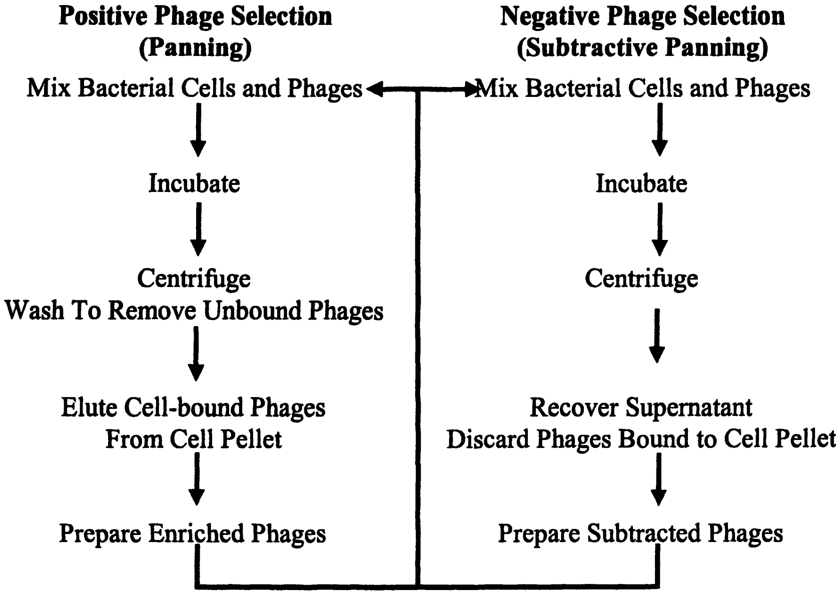
### Selection of an *L. monocytogenes*-Specific scFv

Recently, the author has employed phage display to generate specific antibodies for the detection of *Listeria monocytogenes* (*27*), a foodborne pathogen for which species specific antibodies have been difficult to obtain by traditional methods (*28-33*). Using a whole cell biopanning procedure, the author was able to select phage antibodies that bind to *L. monocytogenes* but do not bind to other species of *Listeria* (*27*). This was accomplished by a series of positive and negative (subtractive) rounds of selection (panning) (Figures 4 & 5). To select phage antibodies that bind to *L. monocytogenes*, cells of *L. monocytogenes* were incubated with the phage expressing antibodies on their surface, non-binding phage were removed by washing, and binding phage were eluted by reducing the pH of the solution (Figure 4). For removal of non-specific phage antibodies (i.e., phage that bind to other species of *Listeria*), the enriched phage preparations were incubated with *L. innocua* and *L. ivanovii* cells. Non-specific phage antibodies bound to the cells of the other *Listeria* spp. and were removed by centrifugation (Figure 4). The supernatant contained phage antibodies that bind specifically to *L. monocytogenes*. Panning schemes involving 4 rounds of positive selection or 7 rounds of panning with subtractive pannings in rounds 4 and 6 were employed (Figure 5). An increase in phage affinity for whole cells of *L. monocytogenes* (as measured by ELISA) was observed with sequential rounds of panning with a dramatic increase after the fourth panning. When the phage pool from the third panning was panned against *L. ivanovii* and *L. innocua* to subtract phage displaying antibody fragments bound to cell surface antigens that were not unique to *Listeria monocytogenes*, a modest drop in affinity was observed. Subsequent rounds of panning using *L. monocytogenes* resulted in an increase in affinity for the subtracted phage pool that was comparable to that of the phage pool derived from the positive panning scheme. The nucleotide sequence of the 6 clones isolated by the positive panning and 5 clones from the subtractive panning scheme showing the highest binding affinity was determined. Each of these clones was identical; therefore, only one clone (monoclonal phage antibody P4:A8) was used for all subsequent experiments.



Positive Phage Selection
(Panning)
Negative Phage Selection
(Subtractive Panning)
Mix Bacterial Cells and Phages
Mix Bacterial Cells and Phages
Incubate
Incubate
Centrifuge
Wash To Remove Unbound Phages
Centrifuge
Elute Cell-bound Phages
From Cell Pellet
Recover Supernatant
Discard Phages Bound to Cell Pellet
Prepare Enriched Phages
Prepare Subtracted Phages

*Figure 4. Positive and negative selection of phage antibodies using whole bacterial cell*

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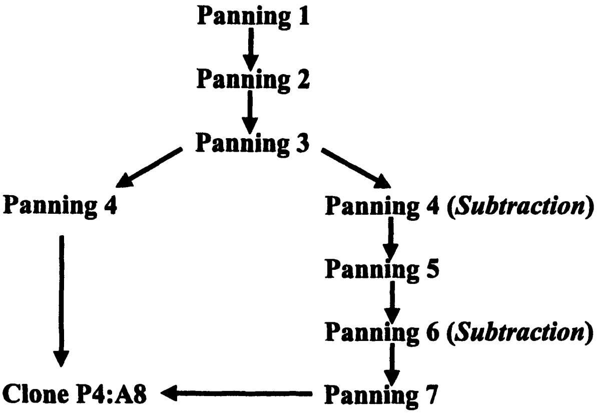

*Figure 5. Panning scheme employed for selection of L. monocytogenes-specific phage antibody P4:A8.*

A collection of bacterial strains was examined to determine the binding-specificity of phage antibody P4:A8. The organisms that were screened included 8 strains of *L. monocytogenes*, a strain of each other species of *Listeria*, and representative Gram-positive and Gram-negative bacteria from other genera. As determined by ELISA, phage antibody P4:A8 showed absolute specificity for *L. monocytogenes* (Table I). Clone P4:A8 exhibited binding to six of the eight *L. monocytogenes* strains and no cross-reactivity toward any of the other species tested. The *L. monocytogenes*-specific antibodies and antibodies selected against other targets can be used in biosensors for the rapid detection of a variety of foodborne pathogens and/or protein toxins.

## Development of biosensors from phage displayed antibodies

Development of immunobiosensors from phage displayed antibodies requires that the selected antibody fragments be modified to act as either capture or detection reagents. As mentioned above, the selection of a phage antibody results in the isolation of the gene encoding the scFv. Thus, the scFv gene can be cloned to generate fusions to useful reporter proteins eliminating the need to produce antibody conjugates. Plasmid vectors have been constructed that allow the fusion of scFvs to bacterial alkaline phosphatase (*34-36*) and green fluorescent proteins (*37-39*). These fusions proteins have been used in immunologically-based detection via enzyme-linked immunosorbant assays (*20, 22*) and flow cytometry, respectively, as well as other biosensor formats (*16, 17, 25, 40, 41*). In addition, a method for biotinylation of scFvs (*42*) has been developed by fusing the antibody fragments to a biotin activation domain (*43, 44*) and expression of the fusion protein in an appropriate *E. coli* strain.

**Table I. Specificity of Phage Antibody P4:A8 for *L. monocytogenes* as Determined by ELISA**

| *Bacterial species* | *Strain* | *ELISA Response* |
|---|---|---|
| *Listeria monocytogenes* | ATCC 19115 | + |
| *Listeria monocytogenes* | F2365 | + |
| *Listeria monocytogenes* | N3-008 | - |
| *Listeria monocytogenes* | Lm4085 | + |
| *Listeria monocytogenes* | ATCC 19113 | - |
| *Listeria monocytogenes* | ATCC 19114 | + |
| *Listeria monocytogenes* | ATCC 19116 | + |
| *Listeria monocytogenes* | Scott A | + |
| *Listeria grayi* | ATCC 700545 | - |
| *Listeria innocua* | ATCC 51742 | - |
| *Listeria ivanovii* | ATCC 89954 | - |
| *Listeria murrayi* | F4076 | - |
| *Listeria seeligeri* | F4880 | - |
| *Listeria welshimeri* | CF1LP | - |
| *Streptococcus thermophilus* | ATCC 19258 | - |
| *Escherichia coli O157:H7* | B1409 | - |
| *Salmonella typhimurium* | 14028 | - |
| *Lactobacillus plantarum* | ATCC 14917 | - |
| *Lactobacillus bulgaris* | ATCC 11842 | - |
| *Pediococcus acidilactici* | F | - |
| *Campylobacter jejuni* | 81-176 | - |
| *Pseudomonas putida* | KT-2442 | - |
| *Clostridium perfringens* | H6 | - |

# Conclusion

Immunologically-based biosensors are a rapid and reliable alternative to the convention microbiological methods for pathogen detection. These methods rely on antibody molecules for biorecognition. While traditional methods of polyclonal and monoclonal antibody production have been useful in generating antibodies with the necessary affinity and specificity for the detection of many

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foodborne pathogens, antibodies for the detection of some pathogens have been difficult to isolate by these methods. Antibody phage display is a molecular method for antibody selection that may function as an alternative method for antibody production, particularly for those pathogens for which traditional methods have failed. Methods have been developed for incorporating antibody fragments from phage display libraries into existing biosensor formats. The selection of phage displayed antibodies and their incorporation into immunobiosensors will provide a valuable complement to more traditional methods in the challenging field of foodborne pathogen detection.

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## Chapter 5

# Quorum Sensing and Food Safety

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Bacteria use various cell-to-cell signaling mechanisms to control the expression of characteristic survival traits in a density dependent manner, which is designated "quorum sensing". It is generally recognized that regulating such extracellular communication in microorganisms, including those that cause food-borne disease, could lead to a safer food supply. Although historically, autoinducing chemical molecules were first described in a marine symbiont, nearly all microorganisms have since been found to have some kind of signaling system for the transcriptional regulation of genes. The production or activity of the chemical signals can be monitored through multiple coupled reporter assays relying on bioluminescence, detection of expressed promoter-colorimetric enzyme assay gene fusions, cloned fluorescent green protein detection systems, and mutational complementations. The identification of species-specific signals enables the competitive inhibition of quorum sensing in pathogens in or on food using natural or synthetic signal analogues. The chemical complexities of food environments offer challenges to detection, identification, and control of such signaling processes with respect to food-borne bacteria.

In Advances in Microbial Food Safety; Juneja, V., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

## A Diversity of Communication Signals

Low molecular weight chemical signaling in bacteria as a result of high population density has been termed "quorum sensing" (*1*). This chemical signaling, as a form of cell-to-cell communication, regulates various processes associated with the virulence of microorganisms, as well as antibiotic production, biofilm formation, sporulation, and toxin production (*2, 3, 4*). In Gram-negative bacteria, intraspecies signaling involves a broad range of acylated homoserine lactone (AHL) autoinducers designated autoinducer-1 (AI-1) (*5, 6, 7*). A second type of autoinducer, (AI-2), presumed to be a furanone derivative or metabolite found in both Gram-negative and Gram-positive bacteria, serves as a signal among and between different species of bacteria (*8, 9, 10*). A third type of bacterial autoinducer (AI-3) has been recently described in enterohemorrhagic *Escherichia coli* that responds to the eukaryotic host cell hormones, epinephrine and norepinephrine, and that regulates the locus of enterocyte effacement pathogenicity island operon and flagella genes (*11*). Gram-positive bacteria are unique in utilizing small processed oligopeptides for intraspecies cellular communication (*12, 13*). Such intricate communication processes among and between microorganisms at the cellular level enable the regulation of genes and virulence characteristics in selectively closed environments. Some of these environments include foods and to this extent there has been relatively little study of these communication processes or their control and/or applications to food environments.

### Signal Recognition Concerns

Some of the problems encountered in detecting cell-to-cell signaling of microorganisms in foods results from the inadequacies of the methods used in current studies. Historically, the regulation of the *lux* operon resulting in bioluminescence from the concentration dependent detection of autoinducing chemical molecules was first described for the marine symbiont *Vibrio fischeri* (*14, 15*). A closely related free-living marine bacterium, *V. harveyi,* was found to have dual systems, either one of which alone is sufficiently capable of leading to light production by utilizing a phosphorylation/dephosphorylation cascade for signal transduction resulting in the expression of the luciferase regulon genes (*16*). As a model organism for confirming the production of AI-1 or AI-2, reporter strains were constructed from *V. harveyi* that were capable of selectively detecting AI-1 [strain BB886; AI-1 sensor$^+$, AI-2 sensor$^-$] or AI-2 [strain BB170; AI-1 sensor$^-$, AI-2 sensor$^+$] (*6*).

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Although the coupled reporter assays were effective in detecting the production of signaling molecules, questions exist as to their adequacy in measuring autoinducers produced from microorganisms other than *V. harveyi* and whether added metabolites, such as glucose, present in various food environments, influenced signaling in the microorganism being tested or in the reporter stain (*6*). To date, a deletion mutation of AI-2 has not been constructed in the reporter strain, quite possibly due to the role of the signaling molecule in global gene regulation or as an essential intermediate in more than one metabolic pathway of the microorganism. The enzyme, LuxS, although credited as the AI-2 synthetase, actually may have a more important role in the detoxification of S-adenosylmethionine from which AI-2 is secondarily produced as a by-product (*7*). The appeal for AI-2 as a universal quorum-sensing molecule resides in the abundance of *luxS* homologues identified in an array of microorganisms and the ease of luminescence induction in *V. harveyi* reporter cells by culture supernatants from such microorganisms (*17, 18*). However, it has also been recognized that AI-2 has only been confirmed as inducing luminescence in *V. harveyi* (*10*).

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## Communication Among Food-Associated Microorganisms Using AI-2

Using *V. harveyi* BB170 as a reporter strain, AI-2 activity was detected in *E. coli* O157:H7, *Salmonella typhimurium*, and *Campylobacter jejuni* (*6, 9, 19*). The studies were often "background-corrected" to account for AI-2 production by the reporter strain in the absence of supernatant additions from the test microorganisms. The culture medium alone could generate the same levels of bioluminescence as test samples measured as relative light units (RLU) following a sufficient incubation period with the reporter strain of *V. harveyi* (*9, 20*). Medium components could increase light production by the *Vibrio* reporter strain that would not indicate the presence of AI-2 (*21*). Also, cell-free culture fluids from *S. typhimurium* and *E. coli* may not stimulate expression of luminescence in *V. harveyi* without added glucose (*6*). Certainly any requirements for added chemical metabolites using a coupled detection assay for the signaling molecules would create disadvantages for using the reporter system with natural or food environments.

Cell-free culture fluids were analyzed from rumen bacteria using the *V. harveyi* reporter cell assay (*22*; Table I). The AI-2 induction rate in this study was characterized as a percentage of that obtained for a positive control, strain BB152 of *V. harveyi*. The AI-2 measurements ranged from 0.05% for

*Selenomonas ruminatium* ATCC 1256 to 52.78% for *Bifidobacterium fibrisolvens* ATCC 19171 (Table I). Rather than interpreting the results as falling short of the 100% induction expected for the control strain known to produce AI-2, the authors concluded that the studies provided evidence of AI-2 production in the culture fluids from the respective rumen bacteria (*22*).

**Table I. AI-2 Measured from Cell-free Culture Fluids of Rumen Bacteria**

| *Microorganism* | *Medium*[a] | *% Induction of AI-2*[b] |
|---|---|---|
| *Bifidobacterium fibrisolvens* ATCC 19171 | CD | 52.78 ± 0.76 |
| *Eubacterium ruminantium* GA195 | CD | 25.79 ± 8.27 |
| *Ruminococcus flavefaciens* C94 | CD | 36.98 ± 5.15 |
| *Ruminococcus flavefaciens* C94 | M | 21.07 ± 4.74 |
| *Ruminococcus flavefaciens* 83 | CD | 25.27 ± 1.67 |
| *Ruminococcus flavefaciens* 89 | CD | 27.13 ± 0.98 |
| *Selenomonas ruminantium* ATCC 12561 | M | 0.05 ± 0.01 |
| *Streptococcus amylolytica* ATCC 19206 | M | 18.18 ± 1.12 |
| Negative Control 1[c] | CD | 5.01 ± 0.40 |
| Negative Control 2[c] | M | 10.79 ± 3.63 |
| *Vibrio harveyi* BB152 Positive Control | CD, M | 100% |

[a] CD, chemically defined medium + 0.4% (w/v) glucose and 0.4% (w/v) cellobiose. M, mixed medium (CD medium + RGCMS)(*23*).

[b] RLU readings for *Vibrio harveyi* strain BB152 (AI-$1^-$, AI-$2^+$) were used as 100% values for comparison of AI-2 production by all strains in their respective test media.

[c] Negative controls consisted of the test medium alone to which the reporter strain, *Vibrio harveyi* strain BB170 (sensor $1^-$, sensor $2^+$), was then added followed by the standard bioassay conditions.

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A chemically defined medium, as well as a mixed medium without bacterial inoculation, resulted in 5.01% and 10.79% induction of AI-2 production in the coupled reporter cell assay, respectively (Table I). Results were considered positive for AI-2 quorum sensing when stimulation of light showed significant differences ($P<0.05$) compared with the negative controls (*22*). In any given food there may be inhibitory influences from acidulents, buffering agents, carbon and nitrogen sources, preservatives, or other components specific to food that could be permissive toward growth of a microorganism but repressive toward AI-2 detection by these means.

Cloak *et al.* (*24*) examined growth and AI-2 production in *C. jejuni*, *C. coli*, *S. typhimurium*, and *E. coli* O157:H7 grown in foods such as milk, chicken broth, or brucella broth (Table II). The results obtained were difficult to interpret as a consequence of multiple variables collectively influencing the bioluminescence measurements. *C. jejuni* grew best in milk to 6.6 $\log_{10}$ CFU/ml and 100 relative light units (RLU), but produced the highest AI-2 production levels in brucella broth at 3,029 RLU corresponding to a culture concentration of 6.2 $\log_{10}$ CFU/ml (Table II). *C. jejuni* and *C. coli* were equally poor with regards to growth and AI-2 production in chicken broth (Table II). *S. typhimurium* grew to higher cell densities in chicken broth (8.9 $\log_{10}$ CFU/ml) and brucella broth (8.6 $\log_{10}$ CFU/ml) along with higher AI-2 activity levels (1,501 and 1,668 RLUs, respectively) when compared to milk (7.4 $\log_{10}$ CFU/ml and 920 RLUs) (Table II). *E. coli* O157:H7 grew to similar levels in all three foods (7.2-7.4 $\log_{10}$ CFU/ml), whereas AI-2 production levels were 2-fold greater in chicken broth (1,792 RLU) and brucella broth (1,854 RLU) than in milk (822 RLU) (Table II). These results indicated that in addition to AI-2 production in the strains of interest there may be interfering influences, such as metabolic effects of medium constituents on reporter cells, that cannot be discounted. Using a cell-coupled

**Table II. AI-2 Production in Milk, Chicken Broth, and Brucella Broth after 24h at 37°C**

| *Food* | *Microorganism* | *Relative Light Units*[a] | *$Log_{10}$ CFU/ml* |
|---|---|---|---|
| Milk | *C. jejuni* | 99.50 ± 1.21 | 6.6 |
| | *C. coli* | 101.50 ± 2.21 | 6.0 |
| | *S. typhimurium* | 920.00 ± 10.22 | 7.4 |
| | *E. coli* O157:H7 | 822.30 ± 10.01 | 7.2 |
| Chicken Broth | *C. jejuni* | 6.25 ± 0.11 | 1.6 |
| | *C. coli* | 5.95 ± 0.81 | 2.0 |
| | *S. typhimurium* | 1,501.41 ± 12.12 | 8.9 |
| | *E. coli* O157:H7 | 1,792.30 ± 20.99 | 7.2 |
| Brucella Broth | *C. jejuni* | 3,029.00 ± 10.66 | 6.2 |
| | *C. coli* | 3,001.05 ± 20.11 | 6.4 |
| | *S. typhimurium* | 1,668.40 ± 15.60 | 8.6 |
| | *E. coli* O157:H7 | 1,854.30 ± 23.01 | 7.4 |

[a] Results represent the averages ± standard deviations with three replicates per experiment. RLUs were expressed as total luminescence per $10^6$ *Vibrio harveyi* strain BB170 cells per well measured using a computer-controlled luminometer.

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assay required background corrections specific to each test condition to ensure minimal influence on the reporter cells, but doing so added undesired complexity to the interpretation of results (*24*). Specific metabolites and signals could have different effects on the reporter strain as compared with the test strain.

A *luxS* mutant was constructed in the Gram-positive anaerobic spore-forming pathogen, *Clostridium perfringens* (*25*). Although the *luxS* mutant was deficient in AI-2 production using the *V. harveyi* AI-2 reporter assay, the production of alpha-, kappa-, and theta-toxins was only decreased 50% from wild type levels (*25*). Toxin production was not completely influenced by AI-2 cell-to-cell signaling. Although AI-2 production was thought to have some influence on the toxins, it was argued that the regulation of toxin genes in *C. perfringens* was unique and complicated by the involvement of various extracellular and intracellular factors (*25*). Therefore, AI-2 may influence the levels of toxin production in *C. perfringens*, but it is not the sole determinant enabling either toxin production or virulence.

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## Disruption of Cell-to-Cell Communication by Analogue Inhibition

In addition to inducers of cellular activities, there also are inhibitors. An ideal scenario could involve the use of an accepted food component to inhibit the expression of genes coding for virulence characteristics in food-borne pathogens. This suggestion is supported by evidence that a furanone produced by the sea alga, *Delisea pulchra*, inhibited virulence characteristics such as biofilm formation and swarming of *E. coli* (*30*). Farnesol has been shown to inhibit filamentation and biofilm formation in the yeast, *Candida albicans* (*31*). A 2,5-dimethyl-4-hydroxy-3(*2H*)-furanone compound extracted from strawberries is an important aroma compound and deterant to fungal growth (*32*). Further, the naturally occurring furanone, ascorbic acid (vitamin C) found in fruits and vegetables, has antimicrobial properties similar to other known natural inhibitors of quorum sensing (*32*). One study examined the ability of an AI-2 analogue, ascorbic acid, to inhibit quorum-sensing, sporulation, and enterotoxin production in *C. perfringens* grown on ground beef (*26*). The rationale for this study was that earlier work had shown the potential for inhibition of quorum sensing by a variety of natural or synthetic analogues of autoinducing signal molecules (*5, 27, 28, 29*). Although post-translationally modified peptides are believed to play a greater role in cellular signaling for Gram-positive and spore-forming bacteria (*18*), the AI-2 reporter cell-coupled bioluminescence assay was chosen as an acceptable means of measuring interspecies stimuli.

Ascorbic acid (10 to 300 mM) inhibited AI-2 activity in *C. perfringens* cell-free extracts from ground beef as well as spore production after 48 h at 37°C (Table III; *26*). A 2 $\log_{10}$ RLU/$10^6$ reporter cell reduction and 3.7 to 5.2 $\log_{10}$

spores/ml decrease was observed compared to no additions of ascorbic acid (Table III; *26*). However, upon substitution of ascorbic acid with sodium ascorbate, the spore inhibition was an effect of acidic pH on cellular growth, producing lower spore totals (Table III). The effects on enterotoxin (CPE) production were surprising. Ascorbic acid, and to an even greater extent sodium ascorbate, stabilized CPE production (Figure 1). Fortunately, preformed CPE in foods does not create a problem due to its heat lability. Continuing studies are examining the influence of low molecular weight peptides on the control of virulence characteristics of *C. perfringens*.

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**Table III. Total Spore Production of *C. perfringens* Following 24-72 h at 37°C in Sporulation Medium**

| | *Spores ($\log_{10}$/ml)*[a] | *Sporulation (%) Total* | *Initial Culture medium pH*[b] | *AI-2 assay medium pH*[b] |
|---|---|---|---|---|
| Ascorbic Acid (mM) | | | | |
| 0 | 6.83 ± 0.36 | 79.92 ± 7.76 | 7.4 | 6.9 |
| 10 | 1.57 ± 0.67 | 27.26 ± 9.87 | 6.8 | 6.6 |
| 30 | 3.14 ± 0.79 | 59.14 ± 12.89 | 5.5 | 6.3 |
| 100 | BDL[c] | BDL[c] | 4.0 | 4.8 |
| Na-ascorbate (mM) | | | | |
| 0 | 6.83 ± 0.36 | 79.92 ± 7.76 | 7.5 | 6.9 |
| 10 | 4.41 ± 1.21 | 73.97 ± 21.53 | 7.5 | 6.9 |
| 30 | 4.43 ± 1.17 | 77.17 ± 18.96 | 7.4 | 6.8 |
| 100 | 4.38 ± 1.17 | 75.60 ± 17.44 | 7.4 | 6.9 |

[a] One milliliter samples of culture (in triplicate) were taken and immersed in a 75°C water bath for 20 min. Spore totals were counted as the mean viable $\log_{10}$ CFU/ml ± standard deviations after 16 h growth on BHI medium under anaerobic conditions (85% $N_2$, 10% $CO_2$, 5% $H_2$).

[b] Medium pH was measured using a Corning pH meter with pH combination electrode #430 (Corning Incorporated Science Products Division, Corning, NY).

[c] Below detectable limits (<10 CFU/ml) under the conditions used for the experiment. The absence of spores was verified using the Schaeffer-Fulton endospore stain followed by microscopic examination (*32*).

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## Evidence for Gene Regulation Beyond the *Lux* Operon in Food-Borne Microorganisms

Elegant work by Sperandio et al. (*34*) using promoter *lacZ* fusions identified genes in the locus of the enterocyte effacement pathogenicity island of *E. coli* O157:H7, including the adhesin intimin and the intimin receptor, as quorum-sensing regulated genes. The identification of density-dependent genes in enterohemorrhagic *E. coli* appeared to be contradictory as fewer than 50 organisms were thought to be necessary for food-borne illness. Despite the low infectious dose of the pathogen, the authors proposed that autoinducers (AI-2) synthesized by normal intestinal flora could result in the transcriptional activation of *E. coli* O157:H7 virulence genes (*34*). Further work with *lacZ*

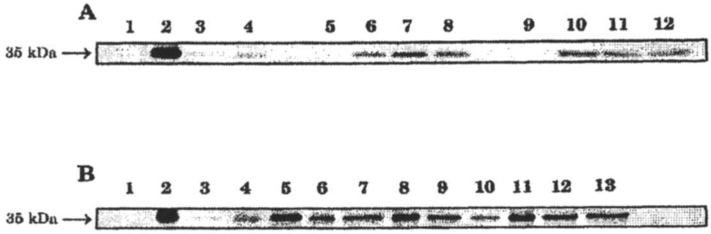

*Figure 1. Western immunoblots of SDS-PAGE gels containing crude cell lysates from C. perfringens cells grown in sporulation medium in the presence of varying A) ascorbic acid or B) sodium ascorbate concentrations over time. Blots were reacted against polyclonal antiserum raised against C. perfringens enterotoxin (CPE). Ten micrograms of total protein were loaded in each well. Arrows indicate location of the 35 kDa antigen. Ascorbic acid concentrations and time of cell harvest in A: Lanes (1) 0 mM after 4 h; (2) 0 mM after 24 h; (3) 0 mM after 48 h; (4) 0 mM after 72 h; (5) 10 mM after 4 h; (6) 10 mM after 24 h; (7) 10 mM after 48 h; (8) 10 mM after 72 h; and (9) 30 mM after 4 h; (10) 30 mM after 24 h; (11) 30 mM after 48 h; and (12) 30 mM after 72 h. Sodium ascorbic acid concentration and time of cell harvest in B: Lanes (1-4) same as lanes 1-4 in A above; (5) 10 mM after 24 h; (6) 10 mM after 48 h; (7) 10 mM after 72 h; (8) 30 mM after 24 h; (9) 30 mM after 48 h; (10) 30 mM after 72 h; (11) 100 mM after 24 h; (12) 100 mM after 48 h; and (13) 100 mM after 72 h.*

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fusions of the regulatory regions of multiple genes in *E. coli* showed that signaling molecules were directly involved not only in controlling genes involved in pathogenesis, but also genes involved in bacterial metabolism, DNA repair, nucleotide and protein biosynthesis, cell division and growth, flagellar motion, and chemotaxis (*35, 36*). The global regulatory mechanism led to the discovery that although LuxS was important in the production of AI-2, inactivation of *luxS* did not prevent quorum sensing by the hormone epinephrine and led to the conclusion that LuxS must be involved in the production of another autoinducer similar to epinephrine, which was described as AI-3 (*11*).

A study was undertaken to examine whether signaling molecules such as AHLs could regulate the production of hydrolytic enzymes, biosurfactants, or lipoproteins leading to the spoilage of preserved foods stored at low temperatures (*37*). The AHLs were detected using a *luxR* bioluminescence monitoring system constructed in *E. coli*, as well as through the use of the *Chromobacterium violaceum* mutant strain (CV026) that could only produce the purple pigment, violacein, upon incubation with supernatants containing AHLs (*5, 37*). It was discovered that AHLs were commonly detected in naturally contaminated samples of vacuum-packaged, cold-smoked salmon containing $10^5$ to $10^7$ *Enterobacteriaceae* per gram (*37*). The study suggested that inhibition of AHL synthesis could give rise to alternate food preservation strategies with respect to lowered use of salts, acids, and antimicrobials (*37*).

A system for monitoring AHL production directly on foods using confocal scanning laser microscopy was described wherein an AHL negative mutant of *Hafnia alvei* carrying the *luxR* gene and *luxI* promoter fused to the gene for green fluorescent protein was then coinoculated with wild-type AHL-producing *H. alvei* (*38*). The visual fluorescence of the AHL-negative reporter cells on meat correlated with the production of AHL by wild-type *H. alvei* (*38*). Using Tn5-transposon mutants of the spoilage organism, *Serratia proteamaculans* strain B5a, the role of quorum sensing AHLs in spoilage of milk was investigated through the proteomic comparison of 2D gels of protein profiles from wild-type strains, AHL production mutants, and mutants complemented with the AHL, 3-oxo-C6-homoserine lactone (*39*). Approximately 39 proteins were identified which specifically play a role in the AHL-regulated spoilage of milk (*39*). Others were similarly able to show that mutants of *Pseudomonas fluorescens* defective in AHL production failed to synthesize extracellular proteases resulting in decreased food spoilage (*40*). *Pseudomonas* spp. have additionally been shown to produce a slimy biofilm facilitated by quorum sensing that accompanied the low temperature spoilage of fresh and ground meats (*41*).

In conclusion, several studies have been addressed in this report with prescribed solutions to regulate food safety through the control of cell-to-cell signaling mechanisms in microorganisms associated with food-borne illness. The

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inhibitory mechanisms could include natural or synthetic signal analogues that interfere competitively with the normal quorum sensing activities in the pathogens. Over 20 bacterial isolates have been identified that produce enzymes that inactivate density-dependent signaling molecules from different sources (*42*). As many of the signaling molecules have been shown to globally regulate many genes and metabolic processes important for a microorganism's survival, the keys to control of food-borne pathogens and food safety appear to be interactive and similar. Crucial to the implementation of biological control strategies will be the development of adequate systems to study the expression of signaling molecules under different food environment conditions and then the application of specific counter measures to foods to inactivate the regulatory systems used by the food-borne pathogens.

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# Chapter 6

# Recent Advances in Pre- and Postslaughter Intervention Strategies for Control of Meat Contamination

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The main approaches employed in controlling microbial contamination in meat products include application of procedures to: (i) minimize sources and levels of microorganisms reaching the slaughter facility; (ii) minimize access or transfer of microorganisms from the animal's exterior and the slaughter environment to the meat; (iii) reduce contamination that has gained access to the meat; (iv) inactivate microorganisms on the meat and meat products; and, (v) inhibit or retard growth of contamination that has gained access to meat and meat products and has not been inactivated. In general, control of microbial contamination on meat products may be accomplished through pre- and post-slaughter intervention strategies. Pre-slaughter or field control of pathogen prevalence in live animals prior to arrival at slaughterhouses may be achieved via good management practices such as market classification of animals, clean housing, feed and water, pest control, and transport/lairage control or via interventions such as diet modifications, feeding of pathogen displacement agents (prebiotics, probiotics, and competitive exclusion), feed additives, antibiotic treatments, vaccine administration, and bacteriophage therapy. Control of

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pathogen contamination on the animal carcass during slaughter and dressing may be achieved through employment of animal cleaning and carcass decontamination technologies, while control at the post-slaughter stage is attained by application of antimicrobial, thermal and non-thermal physical interventions, fermentation, drying, refrigeration or freezing, and antimicrobial packaging. The preservation of meat products is, thus, effectively achieved through combinations of antimicrobial interventions in multiple-hurdle systems. The objective is to maximize the effect of individual antimicrobial interventions in order to achieve an additive or synergistic action that controls contamination more efficiently than the individual treatments applied singly.

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It is well established that foodborne pathogenic bacteria are a major cause of illness and death among humans, accounting for substantial economic losses and suffering. It is estimated that foodborne microbial hazards result in approximately 76 million cases of illness, 325,000 hospitalizations, and 5,000 deaths annually in the United States alone (*1*). The United States National Health Objectives for 2010 aim at reducing the incidence of illness caused mainly by four foodborne pathogens, namely *Campylobacter*, *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*, to 12.3, 6.8, 1.0, and 0.25 cases per 100,000 populations, respectively (*2*). Efforts by the food industry during the last decade have translated into declining trends of infection with cases of *L. monocytogenes* almost reaching the "Health Objectives" initiative of no more than 2.5 cases per million people, and a 42% decrease in *E. coli* O157:H7 cases (*3*).

The production of meat and meat products involves the slaughter of livestock and subsequent processing of raw meat. Muscle tissues of healthy live animals are generally considered sterile and, thus, processing should yield safe meat products provided that the meat is handled safely and processes are applied correctly (*4*). This, however, is not always the case and pathogens have the ability to evade antimicrobial processes and to contaminate our food supply. Meat is contaminated through two major sources, namely the live animal and the processing environment which is also contaminated by the animal and vice versa (*5*). During slaughter, bacteria from the animal hide or gastrointestinal tract may cross-contaminate the underlying and exposed carcass surfaces. Furthermore, cross-contamination may occur from processing tools, equipment, structural components of the facility, human contact and carcass-to-carcass contact (*6*). Although, it can be assumed that some level of microbial contamination of animal carcasses will occur during slaughtering and dressing (*7*), its extent is dependent on the conditions under which animals are reared, slaughtered, and processed (*8*). Thus, the most important factors affecting the microbiological quality of fresh meat are: prevalence of contamination in the animal, hygienic practices, sanitation, product handling and processing, application of decontamination interventions, and conditions of storage and distribution. Levels of microbiological contamination on red meat carcasses may range between $10^1$ and $10^7$ aerobic mesophiles per $cm^2$ (*9*).

The initial microbial flora of animal carcasses usually consists of soil and fecal organisms comprised largely of mesophilic gram-negative and -positive organisms and to a lesser extent of psychrotrophic gram-negative organisms (*10*). The type and extent of meat contamination is highly variable and the microflora usually consists of gram-negative rods and micrococci including *Pseudomonas* spp., *Enterobacteriaceae*, *Acinetobacter* spp., *Alcaligenes* spp., *Moraxella* spp., *Flavobacterium* spp., *Aeromonas* spp., *Staphylococcus* spp., *Micrococcus* spp., coryneforms, and fecal streptococci (*11*). Additionally, lactic acid producing bacteria, *Brochothrix thermospacta*, *Bacillus* and *Clostridium* spores, and enteric viruses may occur in low numbers on fresh meat while yeasts and molds are rarely found (*11*). The most important pathogens associated with meat include *Salmonella*, *Staphylococcus aureus*, *E. coli* O157:H7, *Clostridium perfringens*, *Campylobacter jejuni/coli*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Aeromonas hydrophila* (*10, 12*).

In an effort to facilitate control of potential transferred of pathogens to fresh meat, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) enforces a 'zero tolerance' policy for visible soil on carcasses during slaughter (*13*) and has declared *E. coli* O157:H7 an adulterant in non-intact fresh beef products (http://www.fsis.usda.gov). There is widespread agreement between regulators, educators, consumers, health authorities, research scientists, and the industry that there should be proactive efforts to reduce, eliminate or control pathogens at all stages of the food chain (*14*). In response, and as required by regulation, the meat processing industry has taken an assertive role to comply with consumer demands, customer specifications or criteria, and regulatory requirements by improving operations through the implementation of Hazard Analysis Critical Control Point (HACCP) programs and employment of multiple control interventions throughout the conversion of live animals into meat. Although meat processors strive to produce products with few or no pathogenic bacteria, it should be understood that processing is not conducted under sterile conditions and when the end product is raw meat it cannot be assured of sterility. In its efforts to enhance the microbiological quality of its products, the meat industry has adopted decontamination processes that may include animal cleaning, chemical dehairing at slaughter, spot-cleaning of carcasses before evisceration using knife-trimming or steam-vacuuming,

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spraying, rinsing, deluging or dipping of carcasses with water or chemical solutions before evisceration and before as well as after chilling (*15, 16*). Such interventions were investigated experimentally (*15, 17, 18*) and validated in actual plant settings (*19*). Although progress has been made in the control of contamination, the frequent product recalls from the marketplace, have led the USDA/FSIS (http://www.fsis.usda.gov) to recommend meat operations to consider *E. coli* O157:H7 a food safety hazard that is reasonably likely to occur in fresh beef. Thus, they should re-evaluate their HACCP plans and should establish plant-validated measures for its control as indicated in various government directives, notices and guidances (FSIS Directive 10,010.1/February 1, 1998; FSIS Notice 44-02/November 4, 2002; Proposed FSIS Directives in Federal Register October 7, 2002/Volume 67, Number 194, Pages 62-325-62334; FSIS Guidance for Minimizing the Risk of *Escherichia coli* O157:H7 and *Salmonella* in Beef Slaughter Operations; FSIS Guidance for Beef Grinders and Suppliers of Boneless Beef and Trim Products).

Additionally, there is a major concern with potential *L. monocytogenes* contamination of ready-to-eat (RTE) meat products due to recent outbreaks and highly publicized recalls of such products. For this reason, USDA-FSIS proposed a rule on performance standards for the production of processed meat and poultry products (*20*). Furthermore, the USDA-FSIS established a rule to control *L. monocytogenes* in RTE meat and poultry products (*21*). Three alternatives to control *L. monocytogenes* during post-lethality exposure of products were offered: (i) Alternative 1 requires application of a post-lethality pathogen reduction treatment (that may include a chemical) and an inhibitory antimicrobial agent or process to control *L. monocytogenes*; (ii) Alternative 2 requires application of either a post-lethality treatment or an antimicrobial agent or process; and, (iii) Alternative 3 requires no application of a post-lethality treatment, but instead requires the combination of a sanitation program with microbiological testing of food contact surfaces and holding of products when positive testing results occur (*21*). In an effort to assure RTE meat product safety and consumer confidence and to meet regulatory requirements, there has been continued development of effective control measures in the processing of such products (*22, 23, 24*). Contamination of RTE meat products occurs primarily during post-lethality exposure to the environment (during peeling, slicing, repackaging, etc.). For his reason the new rule requires control strategies to be applied after the lethal thermal process in the production of RTE meats that support pathogen growth during subsequent product storage. Post-lethality physical treatments that are effective in controlling *L. monocytogenes* contamination applied as pre-packaging treatments on RTE meats include radiant heating and flash steam heating, or as post-packaging treatments, include steam pasteurization, immersion in hot water, ionizing radiation, cycles of vacuum-steam, and high hydrostatic pressure. Inclusion of lactate and diacetate

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combinations in the formulation is a common practice, while additional antimicrobials (benzoates, sorbates, glucono-delta-lactone, nisin, organic acids and their combinations) included in the formulation and/or applied as dipping/spraying solutions before packaging have also been shown to be effective in controlling *L. monocytogenes* on RTE meats (*25*).

There has been a substantial amount of activity recently involving investigation for controlling and reducing microorganisms, and especially pathogens, in the livestock prior to slaughter. The rationale is that reduction of pathogen populations on animals pre-slaughter will lead to a reduced probability of introducing such pathogens at subsequent steps in the process and will enhance the effectiveness of subsequent interventions in the slaughtering and further processing of meat (*25*). Most meat processors employ more than one decontamination intervention, in sequence, and it seems logical that this approach, termed "multiple-hurdle technology" (*26*), can effectively be applied throughout the food chain including the pre-slaughter sector. Thus, as indicated, it should be recognized that control of food safety risks should be based on an integrated approach that addresses all sectors, from the producer through the packer, processor, distributor, retailer, food service, and consumer. The best strategy for improving the microbiological quality of meat is applying technologies that: (i) reduce sources and transfer of contamination on the live animal; (ii) minimize the access and transfer of microorganisms to the product (carcass or meat); (iii) reduce the contamination that has gained access to the product; (iv) inactivate microorganisms in products; and, (v) prevent or control growth of microorganisms which have gained access to the meat or meat products and have not been inactivated.

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## Pre-Slaughter Intervention Strategies

### Management Practices for Control of Pathogens in Live Animals

Although studies have evaluated the effect of management practices on pathogen prevalence in the live animal, data are relatively inconclusive in identifying any such factor that results in a consistent and predictable decrease in pathogens. It is believed, however, that even if management practices do not result in direct reduction of pathogen prevalence in animals, they may control contamination levels in the environment which over time may translate into reduced levels in the animal, water, other food products of plant origin, or animal-to-human transmission (*27*). The following are suggested management factors that may enhance pathogen prevalence and level reductions in live animals (*28*).

### *Market Classifications*

Dargatz et al. (*29*) indicated that the prevalence of *E. coli* O157 may be lower in heavier/older cattle. Other research (*30*), suggested that there was no difference in *E. coli* O157 prevalence in cows of different ages or between cows and their calves one week postpartum (*31*). It is generally thought that separation of animals into distinct age groups should not affect the prevalence of *E. coli* O157 (*32*).

### *Clean Housing*

The association of pen density (number of cattle in a pen) and fecal shedding of *E. coli* O157:H7 in cattle has been shown to be negative; that is, the more the cattle in the pen, the less likely they were to be shedding the pathogen (*33*). Additionally, data have suggested that pen cleaning does not necessarily reduce the long-term presence of *E. coli* O157:H7 (*34*). Further control has also been suggested for manure potentially containing pathogens. The practice of spreading manure onto fields to serve as crop fertilizer may introduce the pathogens on other RTE foods such as fruit and vegetables. Control of contamination in manure may be achieved with a carbonate treatment (*35*) to kill foodborne pathogens; however, it is suggested that the use of ammonia and carbonate in combination may be a more effective method for killing pathogens (*36, 37*).

### *Clean Water*

Although, there has been evidence (*32, 38, 39*) that aggressive and frequent cleaning of water troughs did not affect *E. coli* O157:H7 prevalence in cattle, Shere et al. (*40*) demonstrated that water can be a means of disseminating the pathogen to susceptible herds. In addition, presence of the pathogen in water troughs of feedlot cattle was correlated with animal prevalence (*33*); however, it is uncertain if the presence contributed to increased within-pen transmission or simply an increased likelihood of a positive animal contaminating the water. Thermal inactivation, UV irradiation, chlorine, ozone and electrolyzed water treatments have been considered in reducing *E. coli* O157:H7 in water (*41*). Use of sodium caprylate has been proposed for controlling pathogens in drinking water and is still under investigation.

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### *Clean Feed*

*Escherichia coli* O157:H7 was found in prepared feed in feed bunks; however, no association was established between presence in feed and animal-level prevalence (*33*). More recently, Davis et al. (*42*) found that 0.2% of component feed and 0.4% of feed-mill samples were positive for *E. coli* O157:H7.

### *Diet Modifications*

In addition to keeping the feed source clean, other strategies to control microorganisms in the animal's gut may involve alterations in the diet. Manipulation of diet and feed supplements have been examined for their effects in reducing *E. coli* O157:H7 carriage and shedding in cattle (*43*). The switch from high-concentrate feeding to high roughage has been proposed as a possible control strategy; however, conflicting reports (*32, 34*) make such practices inconclusive and may be ill-advised due to potential adverse effects on animal performance. Younts-Dahl et al. (*44*) observed feeding cattle whole cottonseed (15% of total feed composition) resulted in animals with lower *E. coli* O157:H7 prevalence, while the use of barley in cattle feed has provided indications of increased shedding of *E. coli* (*45*) and *E. coli* O157:H7 (*46*) in cattle. Use of ionophores as feed additives has been suggested for controlling pathogens in the rumen of cattle; however, Edrington et al. (*47*) found it to have little effect on populations of *E. coli* O157:H7 and *Salmonella* in ruminant fluid. Feed additives such as Tasco 14™ (a brown seaweed extract) have been effective in reducing the levels of *E. coli* O157:H7 on cattle hides and in feces (*48*). Alternatively, caprylic acid has provided evidence of reduction in *E. coli* O157:H7 in bovine rumen fluid and may assist in reducing pathogen carriage in cattle (*49*).

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### *Pest Control*

The major concern with pests, especially in the feedlot setting, is their possible influence in spreading pathogens. Research has shown that flies have tested positive for *E. coli* O157:H7 (*32*) and that genotypes of the pathogen strains were the same as those found in cattle (*50*). Although, the role of fly control in reduction of pathogen prevalence has not yet been determined, the following management strategies may afford some control (*28*): (i) habitat changes such as drainage of standing water, bait traps, scraping pens, removal of uneaten food, water trough maintenance, manure composting etc.; (ii) biological control through parasitic wasps, *Bacillus* spp.; and, (iii) chemical control using foliar application or insecticide baits.

### *Transport/Lairage Control*

Research has indicated that indeed transportation from the feedlot setting and lairage at the slaughter facility may influence pathogen prevalence in or on other animals due to increased shedding during transport (*51, 52*). Animals being transported and held prior to slaughter may serve as sources of cross-contamination; however, there is limited opportunity for control of animal-to-animal contamination transfer. Thus, it is necessary to control the condition of such environments to limit the contribution the vehicles and pens make as sources of contamination. Schmidt et al. (*53*) reported that cleaning and disinfection of swine pens during lairage reduced *Salmonella* contamination; however, the effect on actual prevalence is uncertain. Bach et al. (*54*) suggested that cattle should be preconditioned (vaccinated and weaned at 29 and 13 days, respectively) prior to haul to minimize the stress effect of such procedures prior to that afforded by transport. Control of pathogens during transport/lairage may be gained by: (i) cleaning and disinfecting surfaces of trailers prior to animal loading; and, (ii) cleaning and disinfecting holding pens at the slaughter facility (*28*).

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## Antimicrobial Interventions to Control of Pathogens in Live Animals

### *Prebiotics, Probiotics, and Competitive Exclusion*

A strategy that may have potential for the control of foodborne pathogens in animals is feeding of beneficial bacteria (*55*) to compete with harmful pathogens in the animal's gut, ultimately decreasing the pathogen load in the live animal and consequently in the feedlot and slaughter environments. Strategies that involve feeding of biological substrates or organisms must be differentiated. The use of a mixture of undefined microorganisms to control foodborne pathogens in livestock is typically termed competitive exclusion, while the feeding of individual or combinations of specific microbial strains may be termed probiotic or direct-fed microbial treatment (*55*). In addition, the use of prebiotics – carbohydrate substrates that selectively stimulate one or a limited number of commensal bacteria – may be applied to displace pathogens incapable of prebiotic metabolism (*56*). Research has shown that use of bacteria such as *Lactobacillus* spp., *Streptococcus bovis*, *E. coli*, *Proteus mirabilis*, *Enterococcus* *faecalis*, *Pediococcus acidilactici*, *Propionibacterium freudenreichii*, and *Leuconostoc* spp. (*55, 57, 58, 59, 60, 61*) is effective in reducing fecal shedding of *E. coli* O157:H7 in cattle, and use of *E. faecalis*, *S. bovis*, *Clostridium* spp., and *Bacteroides* spp. in reducing *Salmonella* in swine (*62*).

### *Feed Additives and Antibiotics*

Research has indicated that supplementation of sodium chlorate as a feed additive or in the water of cattle (*63, 64, 65, 66*), sheep (*67*), and swine (*68*) effectively reduced pathogen populations in the animal's rumen and consequently in their feces. Sodium chlorate is, however, not approved for use in animal diets. Antibiotics are commonly incorporated into animal rations to treat illnesses, however, some antibiotics affect intestinal populations of pathogenic bacteria (*69*). Use of antibiotics such as tilmicosin, neomycin sulfate, and oxytetracycline has thus far provided evidence of effectiveness in reducing *E. coli* O157:H7 in cattle (*41, 70, 71, 72, 73, 74*). There are, however, concerns related to the use of antibiotics as feed additives due to the potential for foodborne pathogens to develop antibiotic resistance. Such antibiotic-resistant pathogens may enter the food supply and subsequently be transmitted to humans; the result of which renders antibiotic therapy potentially ineffective in combating human illness (*75*).

### *Vaccine Administration*

The development of anti-pathogen vaccines for use in animals is a relatively new strategy and there is currently intensive research underway to develop effective vaccines that may be approved. Investigations up to now have indicated effectiveness of vaccines in combating *E. coli* O157:H7 in small scale trials with cattle (*70, 74, 76, 77, 78*). One approach of developing an anti-*E. coli* vaccine has been through stimulation of antibodies that prevent attachment of the pathogen, impeding replication and reducing shedding in the environment (*77, 78*). A similar concept employing the feeding of avian egg yolk anti-O157:H7 immunoglobulin has been proposed to inhibit mobility, attachment, and/or efficient uptake of nutrients to control the pathogen in the intestinal tract of cattle.

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### *Bacteriophage Therapy*

Use of bacteriophages (viruses that target and infect bacteria) in controlling *Salmonella* and *E. coli* in poultry (*79, 80, 81, 82*) has, shown promising as well as conflicting results and further investigation is considered to optimize use of such treatment for reducing pathogens (*83, 84*). Shedding of *E. coli* O157:H7 in calves administered a commercial bacteriophage preparation was less than in those not receiving the treatment (*28*).

## Slaughter/Post-Slaughter Intervention Strategies

In this chapter, discussion of advances during slaughter includes those interventions applied to the carcass surface whereas post-slaughter interventions include those applied during and after fabrication/boning of animal carcasses.

### Interventions Applied During Slaughter

### *Animal Cleaning*

Considering that the hide/skin of the animal is one of the principal sources of contamination for the underlying muscle tissue, it is essential to control the level of contamination on the exterior of the animal prior to entering the slaughterhouse. Interventions to remove fecal material and other debris from the exterior of the animal have involved localized hair removal and washing of the animal with water. Recently, it was suggested that the exterior surface of the animal be rinsed using chemical solutions. A study (*85*) comparing the effectiveness of chemical solutions against bacterial contamination on cattle hides concluded the following trend in decreasing order of effectiveness: 70-90% ethanol > 4-6% acetic and lactic acid > 2% acetic and lactic acid > 0.01-0.04% chlorine > water. Furthermore, cattle hide washing with cetylpyridinium chloride has shown promising results (*86, 87*). Removal of the hair on cattle hides using sodium sulfite (chemical dehairing) is an effective hide intervention reducing the chance of hide-to-carcass contamination with pathogens (*88*).

### *Carcass Cleaning*

One of the earliest methods for carcass cleaning involved the use of water washing applied at different pressures (*89, 90*). Although, washing had the potential to spread contamination from one area to another, it did result in

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significant reductions in carcass contamination. The effectiveness of washing is most likely due to its rinsing effect on loosely associated, but not on bacteria attached to surfaces. Alternatively, carcass cleaning may be achieved by physically cutting/removing visible contaminants rather than washing – a process termed knife-trimming (*91*). The rationale behind such an approach is that removal of visual contamination but it may also result in reduction in accompanying microbial contamination.

### *Thermal Carcass Decontamination*

Increase in water temperature results in enhanced microbiological reduction on carcass tissue (*92*). Thermal decontamination rinses using at least 70°C water are commonly applied in the industry and substantially more effective than lower temperatures in reducing microbiological contamination. Other means of thermal decontamination may include: (i) steam pasteurization™, a process applying superheated steam from potable water (*93*); or, (ii) spot decontamination via steam-vacuuming, a process involving the application of hot water and/or steam and the uptake of residual water and soil via a vacuum (*94*) as an alternative to knife-trimming when visible spots of soil are smaller than 2.5 cm in size.

### *Chemical Carcass Decontamination*

Decontamination spray/wash solutions previously proven effective in reducing carcass contamination include organic acid (acetic, citric, and lactic acid), trisodium phosphate, chlorine or chlorine dioxide, sodium chloride, sodium bisulfate, sodium hydroxide, and ozonated water (*6*). Recent developments in carcass decontamination involve the evaluation or use of new chemical solutions, higher concentrations of existing chemicals (i.e., 5% lactic acid as opposed to 2.5% previously approved), and the incorporation of multiple-hurdle systems applying sequential sanitizing applications during slaughter, dressing, chilling, and before boning. Relatively new treatments that have been proposed, tested, and/or applied in the decontamination of carcasses are acidified sodium chlorite, peroxyacetic acid, cetylpyridinium chloride, sodium metasilicate, and lactoferrin B/lactoferricin/activated lactoferrin (*9*). Recent studies suggest that use of chemicals applied during the spray-chilling process (*95*) and immediately following chilling (*96*) of carcasses may contribute to further control of pathogens. An alternative approach to chemical control of bacterial contamination on the carcass surface currently being researched is that of low dose, low penetration electron beam irradiation on chilled beef carcasses prior to fabrication.

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## Interventions Applied Post-Slaughter

### *Antimicrobials Applied to Fresh Meat*

Recently, there has been substantial investigation into the control of the microbial contamination of fresh meat. The use of chemical antimicrobial agents is limited in fresh meat due to concerns about alteration of its fresh state and the need for label declaration of such treatments or changes. Nevertheless, studies describe the effectiveness of antimicrobial compounds such as lactic acid, polylactic acid, lysozyme, nisin, short-chain fatty acids, lactoferrin, lactoperoxidase system, avidin, plant extracts such as spices and their essential oils, sulfur, and phenolic compounds (*97, 98, 99, 100, 101*) applied to fresh whole muscle.

The preparation of fresh ground meat is an important concern because it is a comminuted product generated from trimmings that may be sourced from different animals. The ground product has a higher likelihood of becoming contaminated during grinding. For this reason the industry has taken initiatives to control microbial contamination in ground meat. Such control strategies include: (i) pH enhancement of the product using ammonia gas (*102*); (ii) incorporation of antimicrobial proteins from porcine leukocytes (*103)*; (iii) incorporation of antimicrobial agents such as nisin, acetic and lactic acid, potassium sorbate, and chelators in an immobilized calcium alginate gel (*104*); (iv) treatment with trisodium phosphate, cetylpyridinium chloride, chlorine dioxide, acidic calcium sulfate, acetic and lactic acid, and ozone singly or in combination (*105, 106, 107*); and, (v) multiple-hurdle decontamination treatments applying hot air, hot water, and lactic acid (*108*).

### *Antimicrobials Applied to RTE Meat Products*

Antimicrobial agents are widely used in the preservation of RTE meat products. The recent directive issued by the USDA-FSIS to control *L. monocytogenes* in RTE meat products has resulted in the proactive incorporation of generally-regarded-as-safe (GRAS) antimicrobial compounds in products. Such antimicrobials include acetates, diacetates, lactates, benzoates, sorbates, glucono-delta-lactone, essential oils, nisin, acidic calcium sulfate with propionic and lactic acid, and their combinations in the formulation or applied as dipping solutions before packaging (*25, 109, 110*). Alternatively, post-lethality treatments such as radiant heat, hot water or steam pasteurization, freezing, and high hydrostatic pressure are applied pre- or post-packaging of product (*9, 111*). Generally, freezing does not effectively inactivate psychrotrophic pathogens such as *L. monocytogenes*; however, freezing combined with an antimicrobial

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agent is very efficacious in controlling such contamination (*111*). Use of organic acids has also been effective against pathogens on beef when applied as a marinade prior to the preparation (drying) of beef jerky (*112*).

Biopreservation of RTE meat products using lactic acid bacteria (LAB) is another proposed approach to enhance the safety of products. These bacteria offer protection to foods against pathogens by production of inhibitory substances such as acetic and lactic acid, acetoin, diacetyl, hydrogen peroxide, reuterin, and bacteriocins (*9*). If approved, biopreservation may be applied by four methods (*113, 114*): (i) addition of a pure LAB culture and consequential indirect addition of antimicrobial metabolites; (ii) addition of crude antimicrobial metabolite preparations; (iii) addition of semi-purified to purified antimicrobial metabolites; and, (iv) addition of mesophilic LAB cultures that may act as a protective culture under conditions of temperature-abuse. Alternatively, use of bacteriophages has been proposed to control pathogens on RTE products by suppressing their growth (*115, 116*).

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### *Thermal Interventions*

Thermal treatments of post-processed RTE meat products are applied either pre- or post-packaging. Pre-packaging thermal treatments including radiant heating (*117*) and flash steam heating (*118*) have yielded positive results in microbial reduction on RTE meats. Post-packaging treatments, including hot water or steam pasteurization (*119, 120*) and immersion in hot water (*121, 122*) have also been effective in reducing microbial contamination and may be more widely used than pre-packaging thermal treatments to minimize the potential for post-process contamination.

### *Non-Thermal Physical Interventions*

Non-thermal physical interventions for meat that are gaining in popularity and are currently being applied commercially are high-pressure processing (HHP), pulse-electric field pasteurization (PEF), and irradiation (gamma and electron beam) (*123*). High hydrostatic pressure (HHP) involves subjecting meat to a hydrostatic pressure in the range of 100-1000 MPa at low or moderate temperatures which disrupts secondary and tertiary structures of macromolecules and results in damage of microbial membranes (*123*). This technique is advantageous as it provides an alternative to thermal processing without markedly altering the sensory characteristics of meat; although, some protein denaturation and textural changes may occur. Pulsed-electric field (PEF) consists of high electric field strengths (10-70 kV/cm) applied in pulses (10-50) for short durations (1-30 ms) to meat between two electrodes, resulting in a membrane potential across the bacterial cell wall in excess of 1.0 V; sufficient to lyse or damage the cell. The main advantage of this technique is that it has little effect on proteins, enzymes, or vitamins. Irradiation incorporates the exposure of foods to ionizing energy and doses of 3-7 kGy are effectively applied to meat to kill vegetative bacterial cells. Advantages of this technique include microbial inactivation or at least irreparable damage and application to frozen meats while the most common disadvantage is the generation of off odors and flavors in meat.

Additional processing technologies under investigation or in developmental stages include sonication (ultrasonic energy), UV light, oscillating magnetic fields (ohmic heating, dielectric heating, and microwaves), and controlled instantaneous decompression (*9, 123*). Sonication of meat refers to the application of ultrasonic pressure waves in excess of 20 kHz that thin microbial cell membranes, cause localized heating and production of free radicals (*124*). The use of UV light to control microbial contamination is thought to be most effective in meat storage rooms and processing areas (*125*.

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### *Antimicrobial Packaging*

Active packaging of meat involves the incorporation of substances that absorb oxygen, ethylene, carbon dioxide, flavors/odors and those that release carbon dioxide, antimicrobial agents, antioxidants, and flavors (*126*). Antimicrobial packaging specifically incorporates antimicrobial agents with polymeric packaging material or biodegradable films and coatings to simultaneously extend shelf life and safety of meat (*127*). Considering that the package surface is in intimate contact with a potentially contaminated meat surface, the inclusion of bacteriocidal or bacteriostatic agents that can be released slowly during storage makes this technology an exciting alternative to direct meat surface application (*127*). Nonedible antimicrobial packaging may contain any food grade additive, some of which include organic acids (acetic, lactic, benzoic, sorbic, or propionic acid), or their anhydrides, bacteriocins, enzymes, and natural antimicrobial compounds from plant extracts (*9*). Edible antimicrobial films may act as a gas barrier against mass diffusion of moisture, gases, and volatiles, as well as serving as carriers of food additives (*128*); however, they pose a unique opportunity to include antimicrobials such as organic acids, bacteriocins, enzymes, short-chain fatty acids, nitrites, essential oils, lactoferrin, and any other edible antimicrobial compound.

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## Chapter 7

# Thermal Treatments to Control Pathogens in Muscle Foods with Particular Reference to *sous vide* Products

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The use of heat to inactivate foodborne pathogens is a critical control point and the most common means of assuring the microbiological safety of processed foods. Therefore, concerns have been expressed about the public-health risks associated with *sous vide* processed foods because the mild heat treatment, to retain the organoleptic attributes, may not ensure proper destruction of pathogenic and spoilage organisms. Manufacturers must be aware of the rate of cooking-induced thermotolerance of pathogens and must take into account this factor when designing safe heat treatments for *sous vide* or other foods that involve minimal heating temperatures. The safety of *sous vide* processed foods cannot rely on only one 'chilled storage' safety factor. Heating processes should be adequate to destroy pathogens or restrict their growth by incorporation of hurdles. Research has assessed combinations of hurdle techniques relative to affects and interactions to improve the margin of safety of *sous vide* foods. Incorporation of multiple hurdles/barriers increased the sensitivity of pathogens to heat, thereby ensuring the safety of *sous vide* processed foods. Further research employing complex multifactorial experiments and analyses to quantify the effects and interactions of additional intrinsic and extrinsic factors and incorporating these data in "enhanced" predictive models are warranted to ensure the microbiological safety of thermally processed foods.

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## Introduction

The growth or survival of potentially life-threatening pathogens in food environments is a significant food safety hazard. The ability of low numbers of certain pathogens to survive or proliferate even when stored under refrigeration or in reduced oxygen atmospheres constitutes a potential public health hazard. Foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year (*1*). Known pathogens cause 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths (*1*). Only a small portion of the foodborne illness episodes are reported and investigated annually, and the pathogens identified comprise an even smaller portion (*1*). Annual economic losses attributed to foodborne diseases associated with medical costs, productivity losses, and business losses due to legal problems may be as large as 5 to 6 billion dollars (*2*). These food safety concerns are magnified because of consumer demands for refrigerated convenient meals, processed using mild heat treatment. This demand has led to a growth in the application of *sous vide* technology to extend the shelf-life and to keep the quality of fresh foods (*3*).

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## The Thermal Destruction of Microorganisms

The use of an adequate heat treatment to destroy pathogenic and spoilage microorganisms is one of the most effective food preservation processes in use today and for decades. Heat treatments designed to achieve a specific lethality for foodborne pathogens is a critical control point in food processing and is fundamentally important to assure the shelf-life and microbiological safety of thermally processed foods. A key to optimization of the heating step is defining the target pathogen's heat resistance. While over-estimating the heat resistance negatively impacts product quality by altering the organoleptic attributes and nutritional qualities of a food, under-estimating increases the likelihood that the contaminating pathogen will persist after heat treatment or cooking.

The use of relatively mild heat treatment (pasteurization) is widely accepted as an effective means of destroying all non-spore-forming pathogenic microorganisms and significantly reducing the number of natural spoilage microflora, thereby extending the shelf-life of such products. The heat is applied at very high temperatures, such as 121 °C or 250 °F (sterilization) for a short time to render food free of viable microorganisms that are of public health concern or capable of growing in the food at temperatures at which the food is likely to be stored under normal non-refrigerated storage conditions. Specifically, the objective of sterilization is to reduce the probability of an organism's survival in a food to an acceptably low level.

## Predicting Pathogen Resistance

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The microbial safety of thermally processed foods depends on ensuring that potential foodborne pathogens likely to be present are killed during heating. The higher the initial microbial population in a food, the longer the processing/heating time at a given temperature required to achieve a specific lethality of microorganisms. Accordingly, the thermal process is designed based on the expected microbial load in the raw product. As such, the heat resistance of bacteria has been historically described by two characteristic parameters, *D*- and *z*-values. In principle, the *D*-value is the time necessary to inactivate 90% of the initial population of microorganisms present in a food at a specific temperature, whereas the *z*-value describes the temperature change necessary to result in a 90% change in the *D*-value. The D- and z-values are used for designing heat processing requirements for desirable destruction of microorganisms in a particular food.

It has long been accepted that when bacteria are killed by heat they die at a constant rate, i.e., via first-order kinetics [Figure 1; (*4, 5*)]. This model of thermal inactivation forms the basis of calculations used in thermal processing and has served the food industry and regulatory agencies for decades. Unfortunately, the microbial cells in a given population do not have identical heat exposure or resistance, and the improbable chance of a heat-sensitive target in one cell determining the death rate of the entire cell population fails (*6*). There have been significant deviations from predicted relationships observed by many researchers using different methodologies (*5, 7, 8*). Frequently a "shoulder" or "lag" period is observed when bacterial populations remain constant or a "tailing" or slower death rate is observed when subpopulations of more resistant bacteria are present (Figure 1).

Presently, there is no satisfactory explanation for the variability in thermal death kinetics. Some investigators have suggested that deviations from linear survival curves result from heterogeneous cell populations (*9*). Possible

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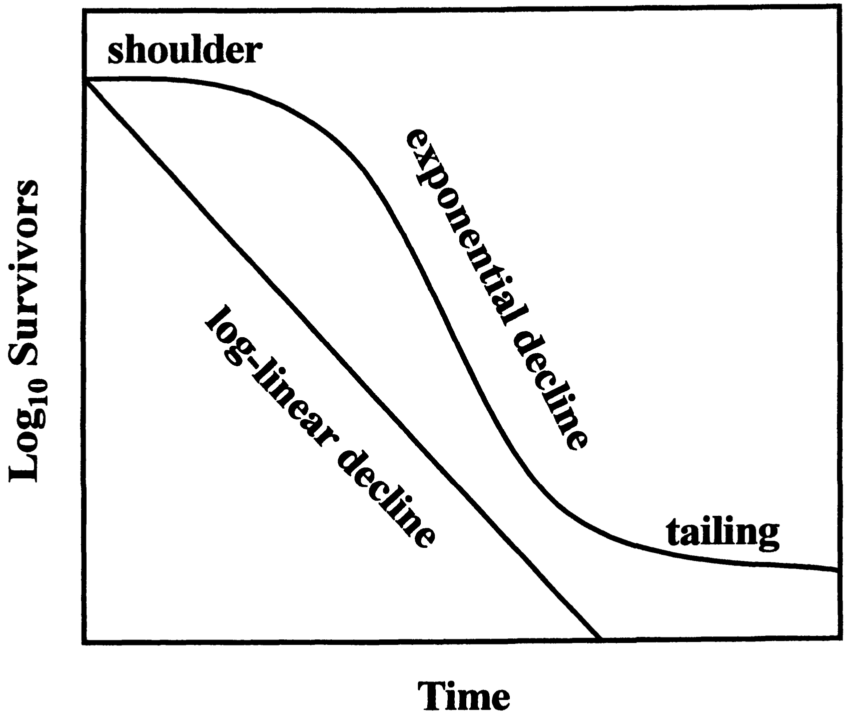

*Figure 1. Thermal inactivation of microorganisms. The straight line represents the traditional first-order kinetics of log number of survivors declining in a linear manner with time. The sigmoidal curve depicts a more realistic representation.*

explanations for the "shoulder effect" include poor heat transfer and the requirement for sufficient cellular injury before observed cell death leads to the expected first-order inactivation relationship. Other theories concentrate on the need for multiple inactivation events or the activation of spores to germinate making them more susceptible to the lethal effects of heat. Clumping of small numbers of cells or spores may protect them against thermal destruction (*4, 9*). In response to environmental conditions or even physiological changes during its life cycle, an individual cell can have varying degrees of heat resistance (*10*). Also, heat resistance can be acquired, as a result of sublethal heating, and lead to deviations from linearity in plotted survival curves. There have been numerous attempts to explain these deviations from the expected linear survivor curves

(*11-15*). Such curves are increasingly observed due to the consumer demand for 'fresh' and ready-to-eat products such as cook/chill, *sous vide* foods. Researchers have expressed concerns about the microbiological risk involved in processing such new generation food products (*16, 17*). Often *sous-vide* foods are prepared by mild heating at low temperatures for long periods of time. Consequently, the survivor curves expected to be encountered for these products may be nonlinear and the inactivation kinetics, accordingly, may be a function of the heating rate. These possibilities demonstrate the need to understand, with greater accuracy, the inactivation rates for determining the safety of foods subjected to mild thermal processes. Practically, it is not feasible to build a large margin of safety in *sous vide* foods that receive a mild, low temperature, long time heat treatment. Therefore, rendering such products completely free of contaminating pathogens by mild thermal processes is a challenge.

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## Factors Affecting Heat Resistance

An appropriate heat treatment designed to achieve a specified lethality of microorganisms is influenced by many factors. Some of these can be attributed to the inherent resistance of microorganisms, while others are due to environmental influences. Examples of inherent resistance include the differences among species and the different strains or isolates of bacteria (assessed individually or as a mixture). Environmental factors include those affecting the microorganisms during growth (e.g., stage of growth, growth temperature, growth medium, previous exposure to stress) and those active during the heating of bacterial suspension, such as the composition of the heating menstruum (amount of carbohydrate, proteins, lipids, and solutes, etc.), water activity ($a_w$), pH, added preservatives, method of heating, and methodology used for recovery of survivors.

## *Sous vide* Food Processing

The French word *sous vide* literally means under vacuum and is a method of cooking whereby fresh food is vacuum sealed in individual packages and cooked (pasteurized) at a time-temperature combination to destroy vegetative foodborne pathogens, spoilage microflora and some spore-formers. After cooking, the products are quickly chilled and then kept in chilled storage (1-4°C), and re-heated prior to consumption. The shelf-life of *sous-vide* products ranges from one to six weeks. For a short shelf-life product (<10-14 days), the significant microbiological risk is the presence of vegetative pathogens, and the heat treatment should achieve at least a 6-$\log_{10}$ reduction (70°C for 2 min in the slowest heating point) in the numbers of pathogens. For longer shelf-life products, the thermal process must eliminate any spores capable of germination and outgrowth during prolonged storage and must be at least equal to 6-D for psychrotrophic *Clostridium botulinum* (90°C for 10 min) or greater if spores of psychrotrophic *Bacillus* species also must be eliminated. It is worth mentioning that milder heat treatments to retain the organoleptic attributes or inadequate processing may not ensure proper destruction of potentially pathogenic and highly heat-resistant bacteria that are non-spore forming. Also, storage abuse of *sous vide* foods could lead to high levels of surviving vegetative or spore-forming foodborne pathogens.

This chapter will deal with the potential threat of foodborne illness through the consumption of *sous vide* processed foods contaminated with spore-forming pathogens, such as *C. botulinum*, *Clostridium perfringens* and *Bacillus cereus* because of their ability to survive the heat treatment given to these products and their subsequent germination, outgrowth, and multiplication during cooling, storage and distribution. Non-spore forming, facultative, psychrotrophic pathogens considered prime hazards in *sous vide* processed products include *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*. These pathogens are capable of growth at refrigeration temperatures under anaerobic conditions (*18, 19*) and hence, pose a potential threat to consumer safety in *sous vide* products. Non-spore forming, mesophilic, facultative anaerobes such as *Salmonella* spp., *Staphylococcus aureus*, or enteropathogenic strains of *Escherichia coli* may also pose a risk if foods are stored at abusive temperatures. All of these vegetative pathogens should be eliminated by the *sous vide* pasteurization step. However, they pose a health risk if the wide variety of raw ingredients used in *sous vide* foods are of poor microbiological quality, or if pasteurization is inadequate to destroy the high microbial load of non-spore forming pathogens. Also, these pathogens are considered as hazards in cases of post-process contamination due to imperfectly sealed *sous vide* packs. Furthermore, these pathogens may be capable of surviving thermal processes designed for the production of these foods if the pathogens are able to synthesize heat shock proteins and, thus, exhibit an induced thermotolerance response. Challenge studies conducted with formulated *sous vide* products to assure safety both from spore-forming and the vegetative foodborne pathogens will be discussed. The effect of heat treatment on the destruction of pathogens and the risk of the surviving pathogens on their growth, and possible toxin production, in case of spore-formers, in *sous vide* foods has been the aim of the research conducted using inoculated pack studies.

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## Justification For Concern

Concerns have been expressed about the microbiological safety and preservation of *sous vide* processed foods. These concerns are justified for a variety of reasons: *Sous vide* products are generally formulated with little or no preservatives and have a low acid and high moisture (high $a_w$) content; these products undergo minimal thermal processing, are not commercially sterile or shelf stable and must be refrigerated; vacuum packaging provides a favorable environment for anaerobic pathogens, such as *C. botulinum* to grow and produce toxin in the processed product while the food remains edible because the spoilage microflora are inactivated; and the potential exists for temperature abuse.

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### *Clostridium botulinum*

The organism that poses the greatest threat to *sous vide* products is *C. botulinum* types A, B, E, and F. Proteolytic type A and B strains of *C. botulinum* are more tolerant to environmental stresses, produce highly heat-resistant spores and have a minimal growth temperature of 10°C. Thus, the spores of proteolytic *C. botulinum* strains will survive the mild heat treatment given to *sous vide* food products. However, these are of limited significance in foods which are properly refrigerated. The non-proteolytic *C. botulinum* strains are less tolerant to stresses, form spores that have reduced heat resistance and can grow and produce toxin at temperatures as low as 3.3°C. Spores of these strains that survive the thermal process would pose a botulism hazard even under proper refrigeration temperatures if a secondary barrier is not present. Therefore, without additional hurdles or barriers, heat processing must be sufficient to destroy non-proteolytic *C. botulinum* spores if the food is to be safe. Lindstorm et al. (*20*) concluded from their studies on the safety of *sous vide* cooked meat products that the extent of heat treatment needs to be carefully examined individually for each product to ensure product safety with regard to non-proteolytic *C. botulinum.*

Challenge studies have been conducted to verify the effectiveness of the combination of hurdles to control *C. botulinum* toxigenesis. Since non-proteolytic *C. botulinum* spores are less heat resistant, it is practically feasible to inactivate these spores by heat. While Juneja et al. (*21*) reported that contaminated turkey should be heated to an internal temperature of 80°C for at least 91.3 min to give a 6 - D process for type B spores, with the inclusion of 3% salt in turkey, 78.6 min at 80°C was sufficient to achieve a 6-D process (*22*). Thus, incorporating low levels of salt while formulating foods and designing a reduced thermal process that ensures safety against non-proteolytic *C. botulinum* type B in *sous vide* processed foods will maintain the desirable organoleptic attributes of foods.

Juneja et al. (*23*) assessed and quantified the effects and interactions of temperature, pH, salt, and phosphate levels in turkey and found that the thermal inactivation of non-proteolytic *C. botulinum* spores is dependent on all four factors. Thermal resistance of spores can be lowered by combining these intrinsic factors. The following multiple regression equation predicts D-values for any combinations of temperature (70-90°C), salt (NaCl; 0.0-3.0%), sodium pyrophosphate (0.0-0.3%), and pH (5.0-6.5) that are within the range of those tested:

$$\text{Log}_e\ \text{D-value} = -9.9161 + 0.6159(\text{temp}) - 2.8600\,(\text{pH}) - 0.2190\,(\text{salt}) + 2.7424\,(\text{phos}) + 0.0240(\text{temp})(\text{pH}) - 0.0041(\text{temp})(\text{salt}) - 0.0611(\text{temp})(\text{phos}) + 0.0443(\text{pH})(\text{salt}) + 0.2937(\text{pH})(\text{phos}) - 0.2705(\text{salt})(\text{phos}) - 0.0053(\text{temp})^2 + 0.1074(\text{pH})^2 + 0.0564(\text{salt})^2 - 2.7678(\text{phos})^2$$

Additionally, Juneja et al. (*23*) developed confidence intervals to allow microbiologists to predict the variation in the heat resistance of non-proteolytic *C. botulinum* spores. Using this predictive model, food processors should be able to design thermal processes for the production of a safe *sous vide* food with extended shelf life without substantially adversely affecting the quality of the product. Representative observed and predicted D-values at 70-90°C of non-proteolytic *C. botulinum* in ground turkey, at various pH levels (5.0-6.5) supplemented with salt (0.0-1.5%, w/v) and sodium pyrophosphate (0.0-0.2%, w/v) are given in Table I.

In commercially prepared *sous-vide* products (beef, chicken and salmon homogenates), sodium lactate at ≥2.4% in beef, ≥1.8% in chicken, and 4.8% in salmon delayed toxigenesis of non-proteolytic *C. botulinum* type B and E for ≥40 d at ≤12°C (*24*). *Sous vide* processing of raw fish, which is known for frequent contamination with *C. botulinum* at high levels, may require specific formulations, as well as specific thermal processing and storage temperature standards to obtain extended shelf-life. Non-proteolytic *C. botulinum* toxin was detected after four weeks in two of 11 commercially available *sous vide* processed products stored at 8°C (*25*). In another study, only two of 16 *sous vide* processed products (the $T_{ref}$ and z values were 82.2 and 16.5°C, respectively; *26*) containing beef, pork, and mixtures of beef, pork, vegetables, rice, and seafood products at the high inoculum level (200, 000 spores / Kg) showed nonproteolytic *C. botulinum* spores and toxigenesis during storage at 8°C. Interestingly, the FoodMicro Model (FMM) predictions for the lethal effect of the thermal process, and the FMM and USDA-Pathogen Modeling Program predictions for the heat inactivation and safe storage time or growth after processing were not in agreement with the observed results in a majority of the

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challenges. This implies that the safety of *sous vide* products has to be carefully evaluated product by product. Time-temperature combinations used in heat treatments should be reevaluated to provide an adequate degree of protection against survival of spore-formers (*27*). The authors suggested assessing the efficacy of additional antibotulinal hurdles such as biopreservatives and organic acids.

**Table I. Observed and Predicted D-Values at 70-90°C of Non-Proteolytic *Clostridium botulinum* in Ground Turkey**

| *Temperature (°C)* | *pH* | *% NaCl* | *% Phosphate* | *D-value Observed (min)* | *D-value Predicted (min)* |
|---|---|---|---|---|---|
| 70 | 6.50 | 0.0 | 0.00 | 57.7 | 66.0 |
| 70 | 6.50 | 1.5 | 0.15 | 40.1 | 46.5 |
| 75 | 6.25 | 1.0 | 0.10 | 39.1 | 42.3 |
| 75 | 6.25 | 1.0 | 0.20 | 32.9 | 38.6 |
| 90 | 5.00 | 0.0 | 0.00 | 5.0 | 6.3 |
| 90 | 5.00 | 1.5 | 0.15 | 3.1 | 4.8 |

Source: Juneja et al. (*23*).

Researchers have assessed the efficacy of added preservatives on inhibiting or delaying the time to toxin production by *C. botulinum*. Maas et al. (*28*) reported that proteolytic type A and B *C. botulinum* spores inoculated in turkey containing 0, 2.0, 2.5, 3.0, or 3.5% sodium lactate and *sous vide* processed (71.1°C) was toxic after 3, 4 to 5, 4 to 6, 7, or 7 to 8 days, respectively, at 27°C. Thus, sodium lactate exhibited an antibotulinal effect which was concentration dependent. Toxin production by *C. botulinum* type A and B spores was inhibited throughout the 42-day storage period at 15°C of reformulated *sous vide* processed (75°C for 36 min) spaghetti and meat-sauce product containing >1.5% (w/w) salt (*29*). It is worth pointing out that none of the above studies discussed the sensory implications, if any, of the hurdles used. Research is required as sensory acceptability may be a limiting factor in practical use.

A number of predictive models have been developed based on multi-factorial design experiments, extensive data collection and analysis. These models quantify the effects and interactions of intrinsic and extrinsic factors and describe the growth responses of spore-formers (*30*, *31*, *32*). Meng and Genigeorgis (*32*) developed the following predictive regression model for the lag phase duration of non-proteolytic *C. botulinum* type B and E spores (inoculum level: $10^2$-$10^4$ /g) in cooked turkey and chicken meats as affected by NaCl

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(0 - %), sodium lactate (0-3%), inoculum (I) and temperature (T) of 8-30°C and their interactions:

$\text{Log }(1/LP) = -2.29-0.123\ (NaCl)+0.22(NaL)+0.439(T)+0.02(T)(I)$ with $R^2 = 0.945$ where T equals square root of temperature

The Meng and Genigeorgis (*32*) study demonstrated that the lag phase can be extended to >38 days at <8°C in the presence of 2% NaL and 1% NaCl, and an inoculum of 100 spores/g. Increasing the NaCl concentration to 2% extended the lag phase to >55 days. At a mild temperature abuse of 12°C, incorporation of 3% NaL and 2% NaCl was required to prevent toxin production for at least 36 days in turkey meat containing 100 spores/g. Such predictive models can be useful in defining microbiologically safe operating practices, such as conditions for a critical control point in a hazard analysis critical control (HACCP) program, or predicting the growth of a microorganism in a new formulation of a product. Food processors can optimize *sous vide* product formulation by the use of these predictive models.

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## Effect of Lysozyme

Since lysozyme is heat stable and is present in a variety of foods, it may remain active in *sous vide* processed products and may, in turn, negatively affect the safety margin of such foods. Researchers have extensively demonstrated that recovery of heated spores is enhanced when lysozyme is supplemented in the recovery medium (*21, 22, 33, 34*). Consequently, an apparent increase in heat resistance is observed. Peck and Fernandez (*35*) concluded from their studies that if lysozyme is present at concentrations up to 50 $\mu$g/ml in a refrigerated, processed food with an intended shelf-life of 4 weeks, and the food is likely to be exposed to mild temperature abuse of up to 12°C, a heat treatment at 90°C for 19.8 min would be required to reduce the risk of growth of non-proteolytic *C. botulinum* by a factor of $10^6$. However, if a longer shelf life is expected, then higher heat treatment in conjunction with better control of temperature, or additional barriers would be required to ensure safety against neurotoxigenesis by non-proteolytic *C. botulinum*. These findings have implications for assessing heat treatments necessary to reduce the risk of nonproteolytic *C. botulinum* survival and growth during extended storage of *sous vide* foods. Further investigations are warranted to determine the effect of lysozyme on the efficacy of recommended heat processes, and especially on its significance in real food systems. Simulating the conditions in *sous vide* processed foods, Juneja and Eblen (*22*) recovered heated nonproteolytic *C. botulinum* type B spores on both Reinforced Clostridial Medium (RCM) with lysozyme and on RCM with lysozyme and the same salt levels as the heating menstruum. When the recovery medium contained no added salt, D-values in turkey containing 1% salt were 42.1, 17.1, 7.8, and 1.1 min at 75, 80, 85, and 90°C, respectively. Increasing levels (2 and 3%, w/v) of salt in the turkey reduced the heat resistance as evidenced by reduced spore D-values. The D-values were 27.4, 13.2, 5.0, and 0.8 min at 75. 80, 85, and 90°C, respectively, when both the turkey and the recovery medium contained 1% salt. Increasing levels (2-3%, w/v) of salt in turkey resulted in a parallel decrease in the D-values obtained from the recovery of spores on the media containing the same levels of salt as the heating menstruum. The authors indicated that the decrease in D-values obtained from the recovery of heat damaged spores on the media with added salt was due to the inability of heat-injured spores to recover in the presence of salt. The heat-injured spores were sensitive to salt in the recovery medium. These data should assist food processors to design thermal processes that ensure safety against non-proteolytic *C. botulinum* type B spores in *sous vide* foods while minimizing quality losses.

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## *Clostridium perfringens*

The temperature range for growth of *C. perfringens* is 6 to 50°C, with a doubling time as short as 7.1-10 min (*36*). Optimum pH for growth is between pH 6.0 to 7.0, and the growth limiting pH ranges from pH 5.5-5.8 to pH 8.0-9.0. While most strains are inhibited by 5-6.5% salt, the organism has been observed to grow at up to 8% NaCl concentration in foods (*36*).

Researchers in recent years have characterized the behavior of *C. perfringens* in *sous vide* cooked foods. The thermal resistance of *C. perfringens* spores (expressed as D-values in min) in turkey slurries that included 0.3% sodium pyrophosphate at pH 6.0, and salt levels of 0, 1, 2, or 3% are shown in Table II. The D-values at 99°C decreased from 23.2 min (no salt) to 17.7 min (3% salt). In a beef slurry, the D-values significantly decreased ($p<0.05$) from 23.3 min (pH 7.0, 3% salt) to 14.0 min (pH 5.5, 3% salt) at 99°C. While addition of increasing levels (1-3%) of salt in turkey (*37*) or a combination of 3% salt and pH 5.5 in beef (*38*) can result in a parallel increase in sensitivity of *C. perfringens* spores at 99°C, mild heat treatments given to *sous vide* foods will not eliminate *C. perfringens* spores. In other words, spores are likely to survive the normal pasteurization/cooking temperatures applied to these foods. In fact, it is not feasible to inactivate the spores by heat. Cooking temperatures, if designed to inactivate *C. perfringens* spores, may negatively impact the product quality and desirable organoleptic attributes of foods are unlikely to be retained. Mild heat treatment given to *sous vide* foods could serve as an activation step for spores. Thereafter, germination and outgrowth of spores, and *C. perfringens* vegetative growth is likely to occur in *sous vide* foods if the rate and extent of cooling is not sufficient or if the processed foods are temperature abused.

**Table II. Mean[a] Generation times, Lag Times and D-values ± Standard Deviation at 99°C of Spore Cocktail of *Clostridium perfringens* Strains NCTC 8238, NCTC 8239 and NCTC 10288 in Ground Turkey which Contained 0.3% Sodium Pyrophosphate at pH 6 and Salt Levels of 0, 1, 2, and 3% Salt**

| *Product* | *Generation Times (min)[b]* | | *Lag Times (h)* | | *D-value at 99°C (min)* |
|---|---|---|---|---|---|
| | 28 °C | 15 °C | 28 °C | 15 °C | |
| Turkey (salt 0%) | 39.4 | 300.0 | 7.3 | 61.6 | 23.2 ± 0.2 |
| Turkey (salt 1%) | 31.3 | 398.8 | 10.6 | 59.6 | 21.3 ± 0.8 |
| Turkey (salt 2%) | 24.2 | 238.2 | 11.6 | 106.4 | 19.5 ± 0.8 |
| Turkey (salt 3%) | 88.5 | nd[c] | 8.0 | nd[c] | 17.7 ± 0.3 |
| Beef (salt 0%; pH 7) | 80.1 | 415.9 | 11.55 | 96.06 | 23.3[b] ± 1.4 |
| Beef (salt 3%; pH 7) | 88.8 | 439.0 | 16.58 | 159.06 | 19.8[b,c] ± 2.1 |
| Beef (salt 0%; pH 5.5) | 122.1 | 4640.7 | 12.83 | 200.52 | 17.3[b,c] ± 0.1 |
| Beef (salt 3%; pH 5.5) | 129.2 | NG | 27.53 | NA | 14.0[c] ± 1.7 |

[a] Mean of two replications.

[b] Generation times calculated from regression lines for exponential growth using the Gompertz equation.

[c] not determined.

Source: Juneja and Majka (*38*); Juneja and Marmer (*37*).

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C. *perfringens* spores germinated and grew at 28°C from 2.25 to >5 $\log_{10}$ cfu/g after 16 h in *sous vide* processed (71.1°C) turkey samples regardless of the presence or absence of salt (*37*). While *C. perfringens* spores germinated and grew at 15°C to > 5 $\log_{10}$ cfu/g in turkey with no salt by day 4, the presence of 3% salt in samples at 15°C completely inhibited germination and subsequent multiplication of vegetative cells even after 7 days of storage (*37*). Growth from *C. perfringens* spores occurred within 6 days in *sous vide* processed (71.1°C) pH 7.0 ground beef samples, but was delayed until day 8 in the presence of 3% salt at pH 5.5 at 15C, (*38*). *C. perfringens* growth from a spore inoculum at 4°C was not observed in *sous vide* cooked turkey or beef samples (*17, 38*). The generation and lag times in *sous vide* processed beef and turkey at 28° and 15°C are given in Table II.

The efficacy of sodium lactate (NaL) in inhibiting the growth from spores of *C. perfringens* in a *sous vide* processed food has been assessed. Presence of 3% NaL in *sous vide* beef goulash inhibited *C. perfringens* growth at 15°C, delayed growth for a week at 20°C, and had little inhibitory effect at 25°C (*39*). While addition of 4.8% NaL restricted *C. perfringens* growth from spores for 480 h at 25°C in *sous vide* processed (71.1°C) marinated chicken breast, it delayed growth for 648 h at 19°C. *C. perfringens* growth was not observed at 4°C regardless of NaL concentration (*40*). These studies suggest that NaL can have significant bacteriostatic activity against *C. perfringens* and may provide *sous vide* processed foods with a degree of protection against this microorganism, particularly if employed in conjunction with adequate refrigeration.

Since *C. perfringens* may grow and multiply rapidly after germination, cooked meat and poultry products must be cooled rapidly to restrict their germination, outgrowth, and the subsequent vegetative growth. To ensure safety, *sous vide* products must be transported, distributed, stored and handled under refrigeration. Juneja et al. (*41*) reported that no appreciable growth (< 1.0 $\log_{10}$ CFU/g) occurred if cooling took 15 h or less when cooked ground beef inoculated with heat activated *C. perfringens* spores was cooled from 54.4° to 7.2°C at an exponential rate, that being more rapid cooling at the beginning and then slower. However, *C. perfringens* grew by 4-5 $\log_{10}$ CFU/g if the cooling time was greater than 18 h. This implies that *C. perfringens* is capable of rapid growth in meat systems, making this organism a particular concern to meat processors, as well as to the foodservice industry.

Predictive bacterial growth models that describe *C. perfringens* spore germination and outgrowth during cooling of food have been generated by researchers using constant temperature data. Juneja et al. (*42*) presented a model for predicting the relative growth of *C. perfringens* from spores, through lag, exponential and stationary phases of growth, at temperatures spanning the entire growth temperature range of about 10°-50°C. Juneja et al. (*43*) developed a predictive cooling model for cooked cured beef based on growth rates of the organism at different temperatures, which estimated that exponential cooling from 51° to 11°C in 6, 8, or 10 h would result in an increase of 1.43, 3.17, and 11.8 $\log_{10}$ CFU/g, respectively. A similar model was later developed for cooked cured chicken (*44*). Smith-Simpson and Schaffner (*45*) developed a model from data collected under changing temperature conditions to predict growth of *C. perfringens* in cooked meat during cooling. Huang (*46, 47, 48*) used different mathematical methods to estimate the growth kinetics of *C. perfringens* in ground beef during isothermal, square-waved, linear, exponential, and fluctuating cooling temperature profiles. These predictive models should aid in

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evaluating the safety of cooked products after cooling and, thus, with the regulatory disposition of products subject to cooling deviations.

## *Bacillus cereus*

*B. cereus* control is a challenge in *sous-vide* processing since the organism is a facultative anaerobe and a spore-former. The organism is recognized as a psychrotrophic pathogen, though the temperature for growth of *B. cereus* ranges from 15-50°C.

The heat resistance of *B. cereus* spores is a concern to the food industry and has been studied extensively. In general, the heat resistance is similar to that of other mesophilic spore-formers; however, some strains, referred to as heat-resistant strains, are about 15-20-fold more heat resistant than the heat sensitive strains (*49*). *B. cereus* strains involved in food poisoning have D-values at 90°C ranging from 1.5 to 36 min. It is most likely that the organism will not be completely destroyed by the heat treatment given to most *sous vide* foods. Therefore, the organism must be controlled in these foods by preventing its growth and/or restricting the shelf-life of the product. As with *C. perfringens*, the risk of food poisoning due to *B. cereus* is relatively low because of the relatively high infective dose which ranges from $10^5$-$10^7$ organisms (total) for the diarrheal type and from $10^5$-$10^8$ organisms per gram of food for the emetic syndrome.

A psychrotrophic *B. cereus* strain survived pasteurization and grew at 7°C in *sous vide* cooked green beans (50). In a challenge study using *B. cereus* in vacuum cooked foods by Chavez-Lopez et at. (*51*), none of the heat treatments applied were able to inactivate *B. cereus* by more than 2 $\log_{10}$ cfu/gm in the tested foods. Counts of this pathogen declined progressively in all products during storage at 4°C and 15°C, except for a transient increase to >6 $\log_{10}$ cfu/g observed in rice pilaf at 15°C. Presumably, these observations might be due to the effect of a particular food component. In another study (*39*), no *B. cereus* growth was observed at 10°C, but after 7 days at 15°C, population densities increased by 1 $\log_{10}$ cfu/g in the *sous vide* beef goulash samples. *B. cereus* populations were reduced by 0.5 to 1.0 $\log_{10}$ cfu/g and by 3 $\log_{10}$ cfu/g in *sous vide* chicken breast heated to 77°C and 94°C, respectively (*52*). These findings suggest that *B. cereus* populations were reduced by the mildest heat; however, the final temperature is important in controlling this organism. In this latter study, spores germinated within 1 day at 10°C, yet detectable changes in populations were not evident through 28 days storage. Baker and Griffiths (*53*) developed a predictive model for psychrotrophic *B. cereus*. The authors used a response surface analysis to determine the effects and interactions of water activity, pH, temperature, glucose and starch concentration on the growth and toxin production by the organism. The authors reported that the factors that had

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the greatest influence on both growth and toxin production were water activity and temperature.

## Vegetative Foodborne Pathogens

While it is universally agreed that proper pasteurization/cooking temperatures will destroy vegetative cells, it is important to ensure that the mild heat treatment designed for *sous-vide* foods provides an adequate degree of protection against vegetative foodborne pathogens. The safety concerns in *sous vide* processed foods relate to the ability of *Listeria monocytogenes* to grow rapidly at refrigeration temperatures and the fact that it is more heat resistant than other vegetative pathogens. *Sous vide* processing of beef and chicken samples to an internal temperature of 70°C resulted in a 4-5 $\log_{10}$ reduction and 70°C/2min resulted in a reduction greater than 7 $\log_{10}$ cycles of *L. monocytogenes* (*54*).

*Sous vide* processed fish is subjected to low time/temperature cooking to retain intrinsic organoleptic attributes. A heat treatment of 90°C for 15 min was the most effective in ensuring the safety of *sous vide* processed salmon with regard to *Staphyloccus aureus*, *Bacillus cereus*, *C. perfringens* and *L. monocytogenes* (*55*). Ben Emarek and Huss (*56*) investigated the heat resistance of two strains of *L. monocytogenes* in *sous vide* cooked fillets of cod and salmon. Pasteurized salmon fillets (10.56 to 17.2%, w/w, fat) had one to four times higher D-values for both strains of *L. monocytogenes* than the lower fat (0.6 to 0.8%, w/w, fat) cod fillets. These findings document the protective effect of fatty materials in the heating medium and the importance of food type on the heat resistance of *L. monocytogenes*.

The slow heating rate/long come-up times and low heating temperatures employed in the production of *sous-vide* cooked foods expose the microbial cells to conditions similar to heat shock, with the possibility of rendering these cells more thermal resistant. Stephens et al. (*57*) and Kim et al. (*58*) have shown that slowly raising the cooking temperature enhanced the heat resistance of *L. monocytogenes* in broth and pork, respectively. Because recovery of heat stressed pathogenic bacteria is increased under anaerobic conditions (*59*, 60), possible growth of heat injured pathogens in *sous vide* products is certainly a concern. Hansen and Knochel (*16*) found no significant difference between slow (0.3-0.6°C/min) and rapid (>10°C/min) heating and the heat resistance of *L. monocytogenes* in low pH (<5.8) *sous vide* cooked beef prepared at a mild processing temperature. However, the latter authors did observe an increase in the heat resistance of *L. monocytogenes* in higher pH (6.2) *sous vide* beef. While processing at slowly rising temperatures may slightly increase the survival of *L. monocytogenes* in cooked beef, there was no evidence of an increase in

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subsequent growth potential of the surviving cells. Therefore, in subsequent studies, Hansen and Knochel (*61*) found that 95-99.9% heat injured *L. monocytogenes* did not grow or repair sublethal injuries in *sous vide* cooked beef at 3°C, while resuscitation and growth took place at 10° and 20°C. All cultures where a high degree of heat injury (≥ 99%) was observed did not subsequently grow in a beef product at 3 or 10°C within 30 days. Quintavala and Campanini (*62*) determined the heat resistance of *L. monocytogenes* strain 5S heated at 60, 63, and 66°C in a meat emulsion at a rate of 5°C/ min compared to instantaneous heating. The D-values of cells heated slowly were two-fold higher than the cells heated instantaneously at all heating temperatures. Thus, varying the rate at which the organism in *sous vide* cooked beef is heated can result in significant changes in the ability of cells to survive a heat treatment at the target temperature. Failure to inactivate *L. monocytogenes* during cooking may lead to an unsafe product even if subsequent transportation, distribution, storage or handling are carried out properly.

Tsuchido et al. (*63, 64*) observed an increase in thermotolerance of *E. coli* by raising the temperature of the cell suspension from 0 to 50°C at various rates prior to holding at 50°C. *E. coli* O157:H7 contaminated *sous vide* processed (55°C in 1 h) ground beef (pH 5.5/lactic acid) should be heated at 55°C, the target temperature, for at least 116.31 min and beef (pH 4.5/acetic acid) for 64.8 min to achieve a 4-$\log_{10}$ reduction of the pathogen; the heating time at 62.5°C to achieve the same level of reduction is 4.39 and 2.60 min, respectively (*65*). Thermal-death-times from this study will assist retail food processors in designing acceptance limits on critical control points that ensure safety of beef contaminated with *E. coli* O157:H7.

Thompson et al. (*66*) increased the thermotolerance of *S. typhimurium* in beef under realistic conditions of constantly rising temperature. Subsequently, Mackey and Derrick (*67*) reported that the heat resistance of *S. thompson*, measured as survival following a final heating at 55°C for 25 min, increased progressively as cells were heated during linearly rising temperatures. In that study, cells were heated at a rate of 0.6 or 10°C per min from 20 to 55°C, and then subjected to a heat challenge at 55°C for 25 min. The authors reported that the extent of induced thermotolerance was inversely related to the rate of heating, i.e., the slower the temperature rise, the greater the increase in resistance.

Deviations from linear survivor curves and increased thermotolerance is of substantial practical importance to food processors in products that are processed by slow heating rate/long come-up times and low heating temperatures (<65°C). Inactivation rates of a cocktail of *Salmonella* spp. in *sous vide* cooked beef exposed to varied "come-up" heating times of zero (control), and one to three hours from 10°C to the processing temperature of 58°C were examined (*15*). The observed survival curves, determined at 58°C, displayed a slight "shoulder"

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followed by non-zero asymptotic D-value. The estimated averages of the asymptotic D-values for the control and one-hour come-up times survival curves were about 5.7 min; whereas for the two- and three - hour come-up times survival curves were 7 and 8 min, respectively. Thus, Juneja and Marks (*15*) characterized asymptotic D-values for *Salmonella* spp. subjected to different heating rates in *sous vide* cooked beef and reported that the rate of heating can substantially influence the heat resistance of *Salmonella* spp. These findings could have substantial practical importance to food processors in *sous vide* cooked beef that are processed by slow heating rate/long come-up times and low heating temperatures.

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## Control of Pathogens and Future Research Directions

The assurance of microbiological safety is a key factor in the success of *sous vide* processed food products. Accordingly, survival of pathogens and the occurrence of temperature abuse throughout distribution, in retail markets and home refrigerators is a challenge for innovative interventions. The safety of *sous vide* processed foods cannot be considered to rely on only one "chilled storage" factor. Since the survival during thermal processing is dependent on the initial microbial load, the microbiological quality of raw materials plays a significant role in ensuring the safety of *sous vide* food products and should not be of poor quality. The most critical step in the production of *sous vide* products is the heating process for the inactivation of pathogens. In addition, every effort should be made to extend the lag and generation time of the pathogens in foods. The combination of heat with hurdle technology has enormous potential to improve the margin of safety of *sous vide* foods. Research has assessed and quantified the effects and interaction of combinations of hurdles in foods. Combining several inhibitory parameters at sub-inhibitory levels, with an aim to render the pathogens more sensitive to the lethal effect of heat and to determine the possibility of pathogen growth during storage has proven effective. Studies that require further investigation on multiple food formulations should be aimed at identifying optimal processing time-temperature combinations and improved microbial safety during storage. This would provide secondary barriers to pathogen growth in cases of temperature abuse or failure of other primary preservative techniques. Further research employing complex multifactorial experiments and analysis to define and quantify the effects and interactions of additional intrinsic and extrinsic factors and development of "enhanced" predictive models is also needed.

In view of the continued interest that exists in lowering the heat treatment, it would be logical to define a specific lethality at low temperatures. It would be useful to determine the possible effects of injury to vegetative cells and spores that may result from mild heat treatments and factors in foods that influence the recovery of cells/spores heated at these low temperatures. Also, future research should focus on conducting dynamic pasteurization (low temperature-long-time cooking) studies to assess the integrated lethality of cooking, and develop integrated predictive models for pathogens for the thermal inactivation, injury, repair and behavior in *sous vide* foods during storage.

These models should be validated by appropriate microbiological challenge studies to ensure safety. These studies involve inoculation of foods with the bacteria of interest and simulating the conditions of any stage(s) from preparation to consumer use. The microflora of foods are then monitored throughout the study to determine the potential safety hazard with the food. It is important that challenge studies be designed specifically for each new product or when an old product with changes in product formulation is to be launched on the market. Other interventions to prevent surviving pathogens from growing in *sous-vide* processed foods may include:

a) Addition of competing microflora: Lactic acid bacteria may be incorporated in foods. These microorganisms can have both direct and indirect antagonistic action on the growth of surviving pathogens. These bacteria may survive processing and grow when there is temperature abuse, and produce acid and bacteriocins, make the product inedible and warn the consumer of possible hazard. However, further research needs to be done in this area.

b) The use of bacteriolytic enzymes: Lysozyme is present in a variety of foods of both plant and animal origin and is relatively heat stable particularly under acidic conditions. The implications on enhanced germination and outgrowth of spores in *sous vide* foods during storage must be considered while designing the heat treatment and assessing the safety of such foods.

c) Use of time/temperature indicators (TTI): Rigorous control of temperature during transportation, distribution, retail storage or handling before consumption is extremely important. Since temperature abuse is a common occurrence at both the retail and consumer levels, producers should not rely on low temperature storage and should use TTI to track the time and temperature history of the products from production to consumption.

d) Education: Workers in food-processing, food-distribution, and food-service establishments should be exposed to continuing training for safe food production including the consequences of temperature abuse of these food products. Additionally, consumers must also be made aware of the potential hazards associated with these products and they must receive adequate knowledge regarding handling and storage of these products.

e) All establishments within the food chain should have Hazard Analysis Critical Control Point (HACCP) systems in place to ensure that safe practices are carried out and pathogen controls are properly executed and maintained during the maximum permitted shelf-life of the *sous-vide* foods. HACCP plans should

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include an adequate heat treatment, that depends upon the rate of cooking designed to kill heat-sensitive microorganisms (e.g., spoilage bacteria, infectious pathogens and some spore-formers) in *sous vide* cooked beef. If the safety of *sous vide* products is to be ensured in the future, then it is critical that all potential hazards for each product are identified and controlled using a HACCP approach. Further, development of quantitative risk assessment models based on product composition/formulation, and processing and storage, in conjunction with implementation of HACCP plans and employee training in HACCP principles, should provide an adequate degree of protection against foodborne spore-formers and non-sporing psychrotrophic pathogens. Also, this approach should result in a higher degree of confidence in product safety than is possible using traditional end sampling approaches to microbiological control. Finally, Good Manufacturing Practices are advocated to enhance the safety of *sous-vide* products.

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## Chapter 8

# Recent Advances in Food Irradiation: Mutagenicity Testing of 2-Dodecylcyclobutanone

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Treatment of foods containing fatty acids, including meat and poultry, can lead to the formation of a class of chemicals called 2-alkylcyclobutanones that are unique to irradiated foods. The major 2-ACB formed in irradiated meat is 2-dodecylcyclobutanone (2-DCB), which is formed by radiolysis of palmitic acid. On average, approximately 6.0 μg of 2-DCB is present in an irradiated, and then cooked, 125 g ground beef patty. The U.S. FDA recommends that indirect food additives consumed in quantities greater than 1.5 μg per day be tested for safety. Because 2-DCB could be consumed at concentrations that exceed the allowable limit for indirect food additives, consumer groups opposed to food irradiation have requested that 2-DCB be tested in appropriate genotoxicity assays. This has taken on added importance since the availability, on a voluntary basis, of irradiated ground beef as part of the National School Lunch Program 2004. In order to address the question of 2-DCB mutagenicity the compound was tested in 4 mutagenicity tests including the Salmonella Mutagenicity Test, the *Escherichia coli* TRP Assay, a 5-flouro-uracil mutagenesis assay, and for the formation of 6-thioguanine resistant mutants in human TK-6 lymphoblasts. No 2-DCB induced mutagenesis was observed in any of the test systems, both with and without exogenous metabolic activation.

In Advances in Microbial Food Safety; Juneja, V., et al.;
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# Introduction

The use of ionizing radiation to improve the microbiological safety of meat and poultry products has been controversial in the United States and other industrialized nations due to the misplaced association of food irradiation with atomic weapons, nuclear waste, and visions of accidents at nuclear power plants including those at Three Mile Island and Chernobyl. Groups opposed to food irradiation are typically opposed to any type of nuclear technology, and cite the increased risk of cancer associated with exposure to ionizing radiation and nuclear contamination even though irradiated foods do not contain nuclear waste and are not radioactive. Although the U.S. FDA approved the sale of irradiated poultry in the U.S in 1993, and irradiated red meat in 1997, irradiated meat and poultry are sold only in a few thousand markets across the nation (*1*). Consumers are simply uncomfortable with the word irradiation. This is despite many years of research that have failed to demonstrate an increased risk of cancer or birth defects with long-term consumption of irradiated meat and poultry in feeding studies using multiple species of animals (*2*). Objections to irradiation of meat and poultry have been voiced with excessive bile following introduction, on a voluntary basis, of irradiated ground beef into the USDA's National School Lunch Program in 2004.

An issue that further increases the negative perception of irradiated foods are chemicals present in irradiated foods known as unique radiolytic products. Exposure of foods containing fatty acids, such as meat and poultry, to ionizing radiation leads to the formation of compounds called 2-alkylcyclobutanones (2-ACBs), which are not detectable in non-irradiated meat products. Formation of 2-ACBs was first reported by LeTellier and Nawar in 1972 following irradiation of saturated triglycerides at an extremely high dose of 60 kGy (*3*). However, it was not before 1990 that a 2-ACB was identified in irradiated food (*4*).

Stevenson et al. reported the detection of 2-dodecylcyclobutanone (2-DCB) in chicken irradiated to a dose of 5 kGy (*4*). 2-DCB is the most abundant of the 2-ACBs in irradiated meat and is formed by cleavage of the acyl-oxygen bond of palmitic acid by ionizing radiation that leads to its cyclization, resulting in a molecule with the same number of carbon atoms as palmitic acid but with an alkyl group in the second ring position ($C_{16}H_{30}O$; FW 238.41) (Figure 1) (*3*).

The amount of 2-DCB formed as a result of irradiation is dose dependent and reflects the fatty acid composition of the food. In chicken fat there is about 3 times more palmitic than stearic acid, whereas in beef the content of palmitic and stearic acid are similar. In chicken 0.15 - 0.75 μg 2-dDCB/g lipid/kGy has been

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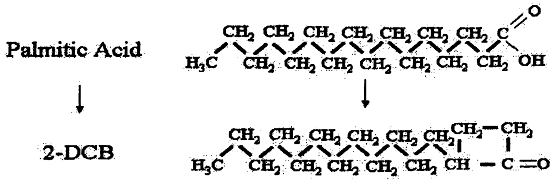
reported (*5-10*) while 0.1 - 0.18 µg/g lipid / kGy, is formed in beef (*11-12*). A person consuming 125 g of cooked irradiated ground beef would be expected to consume approximately 6.0 µg of 2-DCB, or 0.00006 mg/kg for a 100-kg adult, or 0.00024 mg/kg for a 25-kg child (calculated from ref. *13*).



LeTellier and Nawar. (1972) Lipids. 1: 75-76.

*Figure 1. Palmitic Acid and 2-Dodecylcyclobutanone (2-DCB)*

While irradiated meat and poultry has been tested extensively for toxicological safety, unique radiolytic products such as 2-DCB have not been evaluated for safety in pure form. Methodology for the synthesis of 2-DCB, and other 2-ACBs, that could generate sufficient quantities of the compound to complete an appropriate assessment of its mutagenic, clastogenic, and tumor promotion potential has only been recently described (*8*). Even with the ability to synthesize 2-ACBs, including 2-DCB, their high cost (> $12,000/g) has made such testing cost prohibitive (*14*). Despite the high cost and difficulty of testing 2-DCB for genotoxicity Delincee and Pool-Zobel initiated such work in 1998 and found that 2-DCB concentrations of 0.125 to 1.25 mg/ml could induce DNA strand breaks in rat and human tumor cells *in vitro* without the addition of exogenous metabolic activation (*15*). However, these results were later found to be an artifact of the protocol that was used (*16*). The Comet Assay sometimes yields false positive results due to chromosome fragmentation when cytotoxicity (cell death) is induced (*17*). When 2-DCB was retested at non-cytotoxic concentrations, no increase in DNA strand breakage was observed in human colon cell lines (*16*).

Delincee et al. also tested 2-DCB *in vivo* for the ability to induce DNA strand breaks in cells isolated from the colons of rats treated with 2-DCB *in vivo* (*18*). When rats were fed 2-DCB (14.9 mg/kg), a weak genotoxic response (DNA strand breakage using the comet assay) was obtained in rat colon cells (*18*). In the same study, a 2-DCB dose of 1.12 mg/kg did not increase DNA strand breakage in rat colon cells. Based on consumption of 6.0 µg 2-DCB in a cooked 125 g irradiated ground beef patty, a 1.12 mg/kg dose would be the equivalent of a 100 kg adult consuming 18,667 irradiated ground beef patties in one sitting, while a 25 kg child would need to consume 4,667 irradiated burgers

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in one sitting. A 14.9 mg/kg dose, which produced a two fold increase in DNA strand breaks, would be the equivalent of an adult consuming 248,333 irradiated burgers in one sitting, while a 25 kg child would need to consume 62,083 irradiated burgers in one sitting.

Because of the preliminary nature of the results obtained, and the high 2-DCB concentrations needed to elicit DNA strand breaks in the studies, the authors cautioned against interpreting the results to mean that irradiated meats were carcinogenic, and called for further study of the of 2-DCB and other 2-ACBs for genotoxicity. Some groups opposed to food irradiation have claimed, because of the results obtained using the Comet Assay, that 2-DCB is mutagenic (*19-21*). While the claims made by so-called "consumer groups" are at best questionable, current FDA guidelines do, in fact, require safety testing of indirect food additives that could be consumed in excess of 1.5 μg per day (*22*). Although the 2-DCB predicted daily consumption exceeds the 1.5 μg limit, it has never been tested in the battery of FDA "recommended" mutagenicity tests. Because of the controversy surrounding introduction of irradiated ground beef into the National School Lunch program, and the need to provide USDA agencies such as the Food and Nutrition Service and Agricultural Marketing Service, and parents and school district personnel, with an accurate science-based assessment, mutagenicity testing of 2-DCB was initiated at the USDA's Agricultural Research Service, Eastern Regional Research Center, in Wyndmoor, PA.

## Bacterial Mutagenicity Assays

The *E. coli* Trp Assay, developed in 1976 by Green and Muriel (*23*), reports the ability of test compounds to induce reversion of the *trpE65* mutation in *E. coli* from auxotrophy to prototrophy. Reversion of the *trpE65* mutation can occur via a number of genetic pathways (24). The *E. coli* Trp Assay is accepted as a validated short-term genotoxicity test by regulatory agencies of many countries (*25, 26*). The ability of 2-DCB to induce mutations in the *E. coli* Trp Assay (*E. coli* Trp Assay) was examined using the Miniscreen$^{TM}$ version of the plate incorporation test and was found too be negative, meaning that 2-DCB did not induce formation of mutations in the test (*14*). Results of the *E. coli* Trp Assay are listed in Table I.

In the *Salmonella* Mutagenicity Test the strains TA98 and TA 1537 are used to detect induction of frameshift mutations (addition or subtraction of nucleotides in the bacterial chromosome), while TA100 and TA1535 detect the generation of point mutations. 2-DCB did not induce mutations in tester strains TA98, TA100, TA1535 and TA1537, with or without exogenous metabolic activation (5% S9 fraction) as determined by Students *t* test. (n = 3, α = 0.05)

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(Table II) (*27*). Because the *Salmonella* Mutagenicity Test measures induction of frameshift mutations, in addition to point mutations, additional information as to 2-DCB's genotoxic potential, or lack of genotoxic potential, is provided over that of the *E. coli* Trptophan Reverse Mutation Assay. No effect on bacterial viability was observed by examination of the bacterial lawn in the top agar. Results for the negative control (solvent) and positive controls (120 µg/well MMS, 10 µg/well 2-NF, or 10 µg/well 2-AA) were consistent with historical data (*27*).

**Table I. Induction of Mutations in the *E. coli* Tryptophan Reverse Mutation Assay, with or without Exogenous Metabolic Activation, by 2-DCB**

| *Strain* | | *Revertant Colonies per Well*[a] | | | | | |
|---|---|---|---|---|---|---|---|
| *Tester Strain* | *S9 Fraction* | *0 mg* | *0.05 mg* | *0.1 mg* | *0.50 mg* | *1.00 mg* | *Pos. Control* |
| WP2 [pKM101] | 0% | 4.11 | 5.55 | 7.56 | 4.78 | 5.22 | 146 |
| | | ±0.99 | ±1.64 | ±1.75 | ±1.33 | ±1.57 | ±14.7 |
| | 5% | 2.11 | 1.89 | 1.89 | 1.11 | 1.67 | 32.4 |
| | | ±0.11 | ±0.29 | ±0.48 | ±0.40 | ±0.19 | ±0.73 |
| WP2 *uvrA* [pKM101] | 0% | 8.11 | 6.33 | 7.00 | 9.00 | 9.44 | 141 |
| | | ±2.73 | ±3.23 | ±2.52 | ±2.65 | ±3.23 | ±9.45 |
| | 5% | 9.22 | 8.44 | 9.44 | 8.67 | 9.22 | 116 |
| | | ±1.37 | ±2.50 | ±2.50 | ±0.69 | ±0.69 | ±2.67 |

[a] The number of $Trp^+$ revertant colonies per well represents the mean of three independent experiments (n=3) followed by the standard error of the mean for those values (See Ref. *14*).

Bacterial reverse mutation assays including the *Salmonella* mutagenicity test or the *E. coli* TRP reverse mutation assay measure the ability of a xenobiotoc to revert specific mutations in genes required for amino acid synthesis from auxotrophy from prototrophy. In contrast, 5-Flourouracil (5-FU)-resistant mutants in *E. coli* or *Salmonella* are formed when a null mutation is fixed within the DNA sequence of the 0.551 kb uracil-phosphoribosyltransferase gene, which would normally convert 5-FU to a toxic metabolite within the bacterium (*28, 29*). The DNA target available for mutagenesis in these forward mutation frequency assays is much larger than that in bacterial reverse mutation tests, an entire gene as opposed to a point mutation. Sommers and Mackay exposed *E. coli* SF1 to 2-DCB in liquid medium, as opposed to the plate incorporation test. While 2-DCB reduced cell viability to 27% at the highest concentration (1 mg/ml) following a 4 hr incubation period, no increase in mutation frequency (formation of 5-FU-resistant colonies), was observed, with or without exogenous metabolic activation, as determined by Student's *t* test (n=3, α=0.05) (Table III) (*30*).

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## Table II. Induction of Mutations in the *Salmonella* Mutagenicity Test, with or without Exogenous Metabolic Activation, by 2-DCB

| | | *Revertant Colonies per Well*[a] | | | | | |
|---|---|---|---|---|---|---|---|
| *Tester Strain* | *S9 Fraction* | *0 mg* | *0.05 mg* | *0.1 mg* | *0.50 mg* | *1.00 mg* | *Pos. Control* |
| TA98 | 0% | 4.00 | 3.83 | 3.5 | 3.67 | 3.33 | 111 |
| | | ±0.50 | ±0.33 | ±0.00 | ±0.17 | ±0.88 | ±4.16 |
| | 5% | 3.33 | 2.33 | 1.83 | 2.00 | 2.83 | 92.2 |
| | | ±0.44 | ±0.67 | ±0.33 | ±0.29 | ±0.17 | ±3.25 |
| TA100 | 0% | 16.2 | 16.2 | 19.2 | 17.0 | 16.8 | 159 |
| | | ±1.64 | ±1.30 | ±0.67 | ±0.76 | ±1.83 | ±8.26 |
| | 5% | 13.2 | 9.67 | 16.2 | 13.7 | 14.2 | 205 |
| | | ±3.09 | ±1.59 | ±4.32 | ±1.17 | ±3.17 | ±4.49 |
| TA1535 | 0% | 3.50 | 1.50 | 2.17 | 2.83 | 3.50 | 126 |
| | | ±0.29 | ±0.29 | ±0.17 | ±0.17 | ±0.76 | ±4.07 |
| | 5% | 2.16 | 1.17 | 1.50 | 1.67 | 1.33 | 91.2 |
| | | ±0.67 | ±0.44 | ±0.29 | ±0.73 | ±0.44 | ±7.91 |
| TA1537 | 0% | 2.00 | 1.83 | 2.00 | 2.17 | 1.33 | 54.4 |
| | | ±0.29 | ±0.44 | ±0.76 | ±0.17 | ±0.60 | ±5.84 |
| | 5% | 1.17 | 0.50 | 1.67 | 1.00 | 1.50 | 42.8 |
| | | ±0.33 | ±0.29 | ±0.73 | ±0.29 | ±0.29 | ±1.59 |

[a]The number of *HIS*$^+$ revertant colonies per well represents the mean of three independent cultures (n=3) followed by the standard error of the mean for those values (see Ref. *27*).

## Table III. Induction of 5-Fluorouracil-Resistant Mutants in *E. coli* SF1 Exposed to 2-Dodecylcyclobutanone with and without Exogenous Metabolic Activation[a]

| | *Frequency (× $10^{-6}$) of 5-FU resistant mutants* | | | | | |
|---|---|---|---|---|---|---|
| *2-DCB* | *0 mg/ml* | *0.13 mg/ml* | *0.25 mg/ml* | *0.50 mg/ml* | *1.00 mg/ml* | *Pos. Control* |
| No S9 | 0.74 | 1.10 | 0.96 | 0.83 | 0.79 | 12.6 |
| fraction | ±0.17 | ±0.33 | ±0.87 | ±0.16 | ±0.17 | ±2.88 |
| 2% S9 | 1.09 | 0.95 | 0.88 | 0.97 | 0.90 | 7.43 |
| fraction | ±0.14 | ±0.14 | ±0.16 | ±0.14 | ±0.22 | ±1.99 |

[a]Results were tabulated from 3 independent experiments. Positive control compounds were 130 µg/ml MMS without S9 fraction, and 10 µg/ml 2-AA with S9 fraction (see Ref. *30*).

## Mutagenicity Testing in Human Cells

While both the *Salmonella* Mutagenicity Test and the *E.coli TRP* Assay are "recommended" by the FDA, the FDA also "recommends" performing the Mouse Lymphoma Assay, using either Mouse L5178Y tk$^{\pm}$ cells or human TK6 tk$^{\pm}$ lymphoblasts (*22*). 2-DCB was tested for the ability to induce mutations in human TK-6 lymphoblasts to avoid the criticism that human cells were not used.

The genotoxicity assay was carried out using a microtiter plate based method (*31, 32*). TK-6 tk$^{\pm}$ lymphoblasts were purchased from ATCC (Manassas, VA) as were Fetal Bovine Serum (FBS), RPMI-1640 Medium and sterile phosphate buffered saline. 6-Thioguanine (6-TG), HAT medium supplement, and methyl methanesulfonate (MMS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis MO). 2-DCB was purchased from the Fluka subsidiary of Sigma-Aldrich Chemical Co. 2-Aminoathracine (2-AA), S9 Fraction (Aroclor 1254 Induced) and buffers were purchased from Moltox, Inc. (Boone, NC).

TK-6 cells were initially cultured (5% $CO_2$/High Humidity) in RPMI medium containing 10% FBS and HAT medium supplement to suppress the growth of 6-TG resistant cells. The TK-6 cells were then cultured in medium without HAT medium supplement for two passages prior to exposure to 2-DCB or positive control compounds. 2-DCB, MMS (25 ug/ml final conc.), and 2-AA (1.0 μg/ml final conc.) were dissolved in DMSO as solvents. The test compound or positive control compounds were added to 10 ml ($10^6$ cells per ml) of TK-6 lymphoblasts in suspended in cell culture medium in 25cm$^2$ flasks. The total volume of solvent was limited to 1%. The concentration of S9 fraction was 2% in that subset of experiments that required exogenous metabolic activation.

Following a 4 hr exposure to test compounds the cells were pelleted by centrifugation (200 × g) and resuspended in RPMI-40 medium with 10% FBS for 3 days to allow fixation of mutations within the genome and expression of the altered hypoxanthine phosphoribosyl transferase (*hprt*) protein. Following the 3 day recovery period the cells were resuspended and serially diluted in RPMI medium that contained 10% FBS and 1 μg/ml 6-TG to select for *hprt*$^-$ mutants (or the same medium without 6-TG to assess overall viability) and the aliquots transferred to sterile 96 well microtiter plates (150 μl/well). The cells were then cultured for three weeks, and the number of wells that did not contain colonies scored by microscopy. The method of Oberly et al. (*31*) and Sommers et al. (*33*) was used to determine mutation frequency. Each experiment was independently conducted three times. Statistically significant differences were determined using Student's *t* test.

2-DCB did not induce formation of 6-TG resistant mutants in the mouse lymphoma assay using human TK-6 lymphoblasts (See Table IV). Cell viability was reduced to approximately 45% in cultures with and without exogenous

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metabolic activation, as determined by trypan blue exclusion, following the preliminary 4 hr exposure to 2-DCB. Cells treated with concentrations of 2-DCB greater than 0.062 mg/ml were not recoverable following centrifugation, most likely due to severe membrane damage following exposure to the extremely hydrophobic test compound. Increased mutagenesis cannot be ruled out at 2-DCB concentrations that lower cell viability less than the 45% obtained in this study.

**Table IV. Mutation Frequency of TK-6 lymphoblasts Exposed to 2-DCB[a]**

| | *S9* | *0 mg/ml* | *0.018 mg/ml* | *0.036 mg/ml* | *0.062 mg/ml* | *Pos. Control* |
|---|---|---|---|---|---|---|
| Mutation Freq. ($10^{-6}$) | - | 2.78<br>(±0.63) | 3.26<br>(±0.67) | 3.04<br>(±0.11) | 3.20<br>(±0.82) | 26.6<br>(±5.81) |
| Mutation Freq. ($10^{-6}$) | + | 5.04<br>(±0.93) | 6.13<br>(±1.23) | 5.64<br>(±1.66) | 5.55<br>(±1.69) | 55.3<br>(±13.0) |

[a]Positive controls were significantly different from untreated controls as determined Students *t* Test (n=3, $\alpha$=0.05) (This Study).

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## Discussion

The preferred method for assessing the toxicological safety of irradiated foods has traditionally been long-term feeding studies in animals, often for multiple generations. The vast majority of feeding studies failed to find adverse affects associated with consumption from or exposure to irradiated foods. There are, in fact, a small number of studies that produced equivocal results pertaining to the safety of irradiated foods. However, in depth review of those studies determined that they were deficient in experimental design, and used insufficient numbers of test subjects for proper statistical analysis, or experimenter error (*1*).

Some of the more widely known feeding studies in animals include one where rats were fed diets of radiation-sterilized foods for 40 generations and suffered no ill effects from consumption of irradiated foods (*34*). Thayer et al. reported that rodents fed diets of radiation-sterilized chicken meat did not suffer an increased risk of cancer or birth defects (*35*). The same study also failed to find adverse affects associated with long-term consumption of irradiated meat in beagle dogs. Eekelen et al. (*36, 37*) conducted single and multiple generation feeding studies in rats without finding adverse effects due to consumption of the irradiated chicken diet.

However, long-term feeding studies are not designed to evaluate the safety of individual food additives, whether they are added to the food for a specific purpose (direct food additives) or arise as a result of some treatment the food is subjected to (indirect food additives). There is a considerable amount of research pertaining to the testing of food additives created by processing technologies in short-term genotoxicity assays. Mutagenic activity in thermally processed foods has been well established. A number of studies have confirmed the mutagenicity of cooked meats and their fat, and the mutagenicity of nitrosamines formed as a result of cooking (*38-41*). While unique radiolytic products have not been subjected to exhaustive toxicological evaluation, at one time such testing was recommended (*42*).

Very few studies have examined the mutagenicity of 2-ACBs, including 2-DCB, which is clearly present in sufficient quantity in irradiated meat and poultry to qualify as an indirect food additive. In addition to the mutagenicity studies described in this text, Burnouf et al. (*16*) described the testing of multiple 2-ACBs in the Salmonella Mutagenicity test, again with negative results being obtained. Gadgil et al. (*12*) also investigated the ability of 2-DCB to induce mutations in the Salmonella Mutagenicity Test using tester strains TA97, TA98, TA100, TA102, and TA1535, and failed to detect an increase in the formation of mutants as a result of 2-dDCB exposures up to 1 mg/plate. Three laboratories have now failed to detect an increase in mutagenesis as a result of exposure to 2-DCB in the widely used bacterial mutagenicity assays. The remaining 2-ACBs also need to be tested in multiple laboratories in order to arrive at a consensus pertaining to 2-ACB mutagenicity. Of the 2-ACBs, only 2-DCB has been tested in the Mouse Lymphoma Assay using either mouse L5178Y $tk^{\pm}$ cells or human TK-6 $tk^{\pm}$ cells.

2-Dodecylcyclobutanone, a unique radiolytic product of palmitic acid, is present in irradiated ground beef and poultry at low parts-per-million concentrations. 2-DCB was unable to induce mutations in the bacterial mutagenicity tests and in human lymphoblasts, both with and without exogenous metabolic activation when tested in pure (>95%) form. In order to appropriately evaluate the potential genotoxicity of 2-DCB additional experimentation should be conducted to determine the clastogenicity of 2-DCB *in vitro,* and tumor promotion potential *in vivo.*

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## Chapter 9

# Nonthermal Inactivation of *E. coli* in Fruit Juices Using Radio Frequency Electric Fields

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Radio frequency electric fields (RFEF) processing to inactivate bacteria in apple juice at moderately low temperatures has recently been developed. The process is similar to the pulsed electric fields process, except that the power supply is continuous rather than pulsed; therefore, the capital costs may be less. Orange juice and apple cider containing *Escherichia coli* K12 were exposed to electric field strengths of up to 25 kV/cm at frequencies ranging from 21 to 40 kHz. Following treatment at an outlet temperature of 65°C, the population of *E. coli* K12 in orange juice was reduced by 3.4 log relative to the control. Increasing the electric field strength and temperature and decreasing the frequency enhanced the inactivation. The electrical cost of the RFEF processing was approximately $0.0017 per liter of orange juice. There was no change in brownness of orange juice nor was there any loss of ascorbic acid as a result of RFEF treatment. The population of *E. coli* K12 in apple cider was reduced by 4.8 log following RFEF processing at 60°C, whereas, thermal processing at the same time and temperature had no effect. Increasing the electric field strength and temperature enhanced the inactivation; however, there was no enhancement at lower frequency. Scanning electron microscope images of *E. coli* K12 that were thermally processed and RFEF processed indicated that the mechanisms of thermal and RFEF inactivation were dissimilar. The results of the present study provide the first evidence that the RFEF process inactivates bacteria in orange juice and apple cider containing solids at moderately low temperatures.

## Introduction

Outbreaks of food-borne illness caused by contaminated beverages such as orange juice and apple cider still occur despite increased efforts to improve preharvest intervention. Meanwhile, consumers are demanding that these products retain maximum freshness. Hence, nonthermal pasteurization processes are actively being developed. High hydrostatic pressure and ultraviolet light processing have been commercialized to a small extent, but they each have problems which limit their scope. High hydrostatic pressure processing is a batch operation and is much more costly than traditional heat pasteurization. Ultraviolet light processing of opaque juices requires that the juice be formed into a thin film. This restricts the flow rate and the commercial applications (*1*).

High electric field processing has the potential to be commercially adopted on a large scale because it does not suffer from the above problems. It is a continuous operation that is scalable to commercial flow rates. Radio frequency electric fields (RFEF) processing inactivates microorganisms in liquids at low temperatures (*2*). A simple schematic of the process is shown in Figure 1. In this case, a 20 kV/cm electric field strength is produced by separating two parallel plate electrodes by 1 cm and applying a peak voltage of 20 kV to the electrodes. Various other combinations of separation distance and voltage may be used as long as the field generally remains above *5* kV/cm (*3*, *4*). So, for instance, the electrodes could be spaced farther apart in order to accommodate a higher flow rate provided that the voltage increased correspondingly. Other electrode geometries besides parallel flat plates are also possible (*2*, *5*). The voltage can be applied by several different means. In pulsed electric field (PEF) processing, a charging power supply produces a high voltage and a high speed electrical switch delivers the stored energy to the electrodes. The power supply must then be recharged which results in pulsed processing. Bipolar waveforms as presented in Figure 2 are extensively used in PEF processing. In RFEF processing, an AC power supply continuously provides the high voltage as illustrated in Figure 3.

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This potentially simpler method of generating high electric fields may have lower capital and operating costs than those associated with PEF processing.

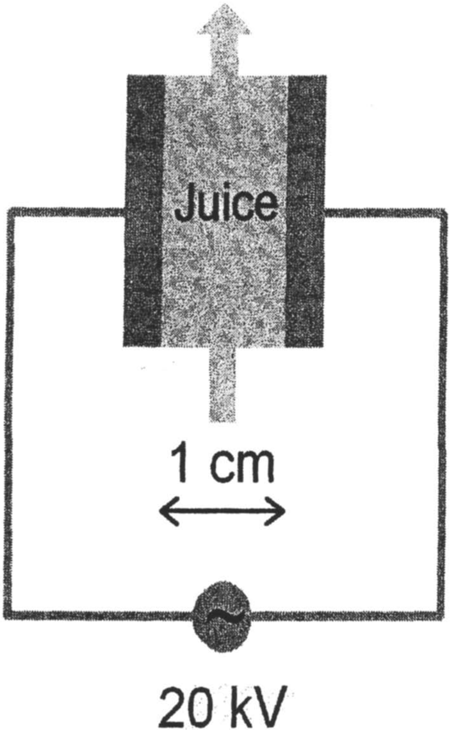

*Figure 1. Schematic of RFEF process. Juice flows between two parallel plate electrodes separated by 1 cm with a 20 kV alternating current across them.*

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Nonthermal inactivation of microorganisms is thought to occur by electroporation (*6*). In an electric field, a voltage is formed across the cell membrane. The opposite charges on either side of the membrane are attracted to each other and the membrane becomes thinner. At a sufficiently high voltage, pores are formed in the membrane and the cell ruptures (*7*).

Nonthermal RFEF processing using bench scale equipment has been shown to be effective at inactivating *Saccharomyces cerevisiae* (*2*) and *Escherichia coli* K12, hereafter referred to as *E. coli* (*8*). Recently, a pilot plant scale RFEF processing system has been designed, fabricated, and assembled (*9*). RFEF processing reduced the population of *E. coli* in apple juice by 2.7 log at 60°C and a hold time of 3 s, whereas conventional heating at the same conditions

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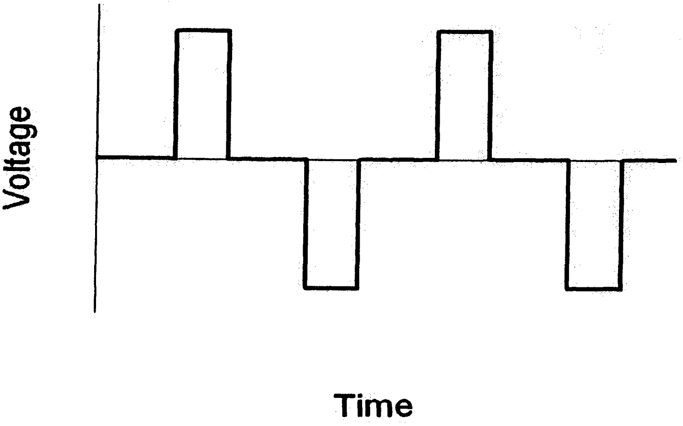

*Figure 2. Example of bipolar pulses used in PEF processing.*

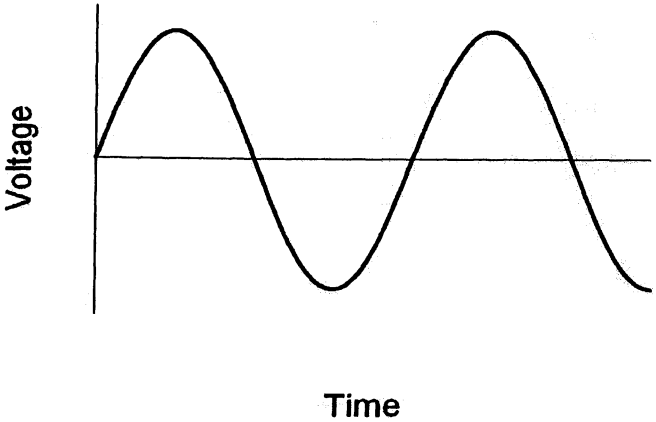

*Figure 3. Example of sinusoidal waveform used in RFEF processing.*

had no effect. The remainder of this chapter will cover RFEF equipment, additional inactivation results, nutritional and quality results, RFEF modeling, scanning electron microscopy results, costs, and the outlook for the future.

## Radio Frequency Electric Fields Equipment

Recently, Geveke and Brunkhorst have developed a pilot plant RFEF process (*9*). The power supply that was constructed consisted of an 80 kW RF power source (Ameritherm, Scottsville, NY, model L-80) and a custom designed matching network (Ameritherm) that enabled the RF energy to be applied to a resistive load over a frequency range of 21.1 to 40.1 kHz (Figure 4). The supplied voltage and current were measured using a voltage divider (Ross Engineering, model VD15-8.3-A-KB-A), current probes (Pearson Electronics, CA, model 411) and an oscilloscope (Tektronix, model TDS224).

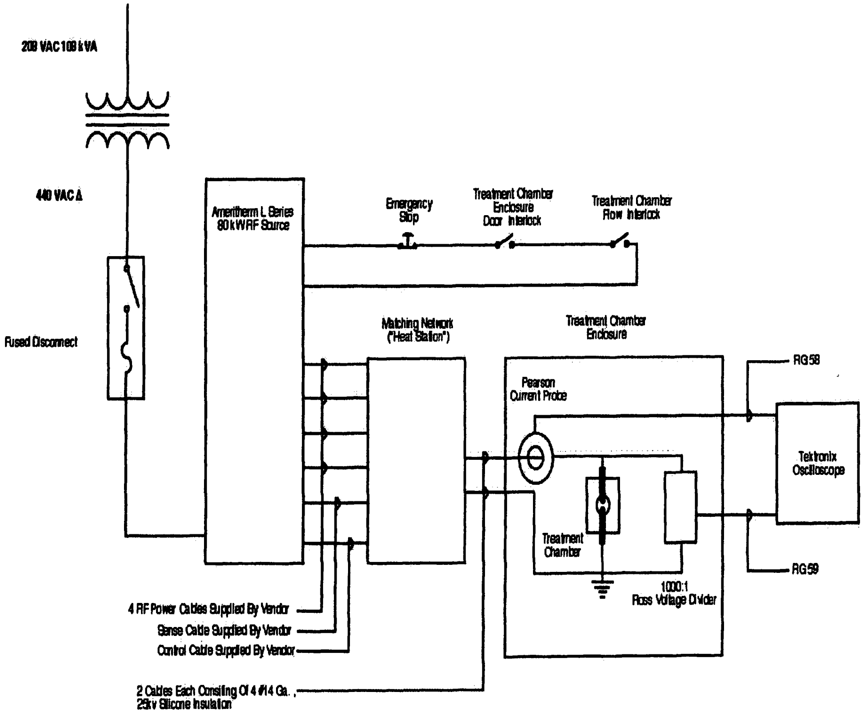
208 VAC 108 kVA
440 VAC Δ
Fused Disconnect
Ameritherm L Series 80 kW RF Source
Emergency Stop
Treatment Chamber Enclosure Door Interlock
Treatment Chamber Flow Interlock
Matching Network ("Heat Station")
Treatment Chamber Enclosure
Pearson Current Probe
Treatment Chamber
1000:1 Ross Voltage Divider
RG58
RG59
Tektronix Oscilloscope
4 RF Power Cables Supplied By Vendor
Sense Cable Supplied By Vendor
Control Cable Supplied By Vendor
2 Cables Each Consisting Of 4 #14 Ga., 25kv Silicone Insulation

*Figure 4. Electrical diagram of 80 kW RFEF system.*

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A novel treatment chamber was designed and fabricated to apply high electric fields to the juices (*8*). The treatment chamber was constructed of Rexolite, a transparent cross-linked polystyrene copolymer (C-Lec Plastics, Philadelphia, PA). It was designed to converge the liquid into a narrow flow area in order to reduce the power requirement (*10, 11*). Liquid entered and exited the Rexolite chamber through the annuli of cylindrical stainless steel electrodes (Swagelok, Solon, OH, part no. SS-400-1-OR) as shown in Figure 5. The electrodes were separated by a thin partition with a channel of circular cross section through the center. The diameter and length of the channel were 1.2 mm and 2.0 mm, respectively, for the experiments done on orange juice. A 9.0 mm space between the end of each of the electrodes and the central channel prevented arcing. For the apple cider experiments, the diameter and length of the central channel were scaled up to 1.4 mm and 2.3 mm, respectively, in order to achieve higher flow rates. It was determined that the space between the electrodes and the channel could be reduced, so as to maximize the electric field, to 2.0 mm without encountering arcing. The output of the RFEF power supply was connected to the electrodes such that the electric flux lines were approximately perpendicular to the direction of the liquid flow.

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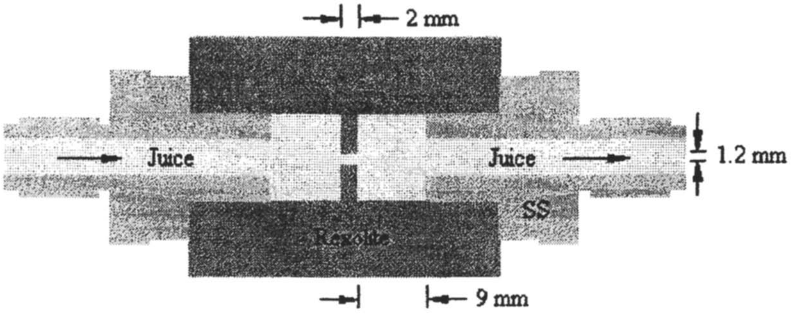

*Figure 5. Cross-section of converged co-field treatment chamber, used in orange juice experiments, including Rexolite insulation and two stainless steel electrodes. The diameter and length of the central channel are 1.2 mm and 2.0 mm, respectively, and the space between the end of each of the electrodes and the central channel is 9.0 mm.*

The treatment chambers can be connected to the RFEF power supply in several different ways. One configuration, that was used for the apple cider experiments, has the cider flowing in series through one or more treatment chambers as shown in Figure 6. The first electrode on each of the treatment chambers is grounded. The remaining electrode on each of the treatment chambers is connected to the RFEF power supply in parallel. Upon exiting the treatment chamber the cider flows through a 1.8 m section of plastic tubing having an internal diameter of 3.2 mm. The purpose of this plastic tubing is to electrically isolate the treatment chamber from the surrounding equipment and ensure that the maximum field is achieved within the chamber. The temperature of the cider rises during RFEF processing due to ohmic (resistance) heating. Therefore, the juice flows through heat exchangers after each treatment to control the processing temperature. Another way of connecting the treatment chambers to the power supply, that was used in the orange juice experiments, is presented in Figure 7. Two chambers are joined by stainless steel tubing. The inner electrodes between the chambers are connected to the RFEF power supply. The outer electrodes are grounded. The advantage of this setup is that there is no concern about isolating the chambers from the surroundings. The disadvantage is that, for a given field, the temperature rise is twice that for a single treatment chamber.

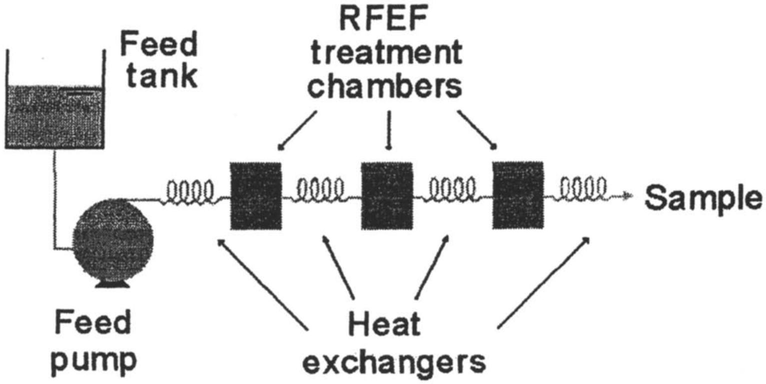

*Figure 6. Schematic diagram of a continuous RFEF process, used in apple cider experiments, including three treatment chambers in series with intercooling.*

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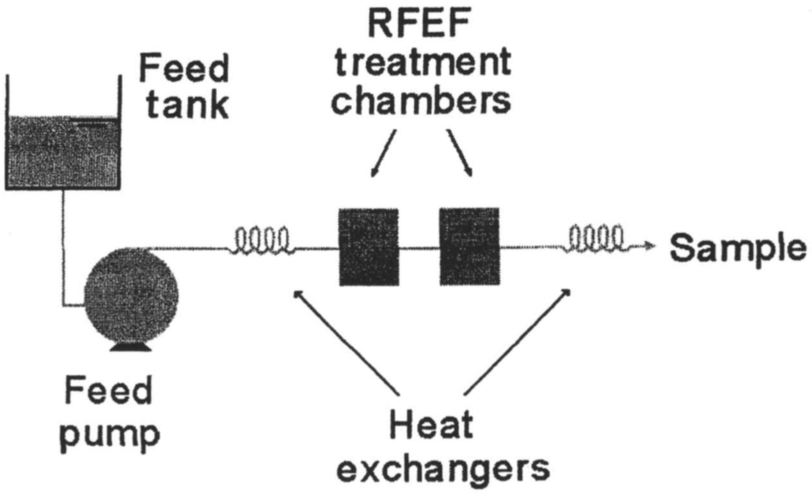

*Figure 7. Schematic diagram of a continuous RFEF process, used in orange juice experiments, including two treatment chambers in series without intercooling.*

The experimental system included a stainless steel feed tank and a progressing cavity pump (Moyno, Springfield, OH; model 2FG3) that supplied the juice to the RFEF treatment chambers at a flow rate ranging of 1.4 to 1.5 l/min. Multiple treatment chambers and turbulent flow within the treatment chambers improved the processing uniformity. The juice was exposed to intense RFEF in each chamber for 110 to 190 μs. At a frequency of 21.1 kHz, the liquid was exposed to at least one complete AC cycle in each chamber. A back pressure of 1 atmosphere gauge minimized arcing. A 0.24 $m^2$ stainless-steel heat exchanger (Madden Manufacturing, Elkhart, IN; model SC0004) and a temperature controller (Cole-Parmer, model CALL 9400) were used to regulate the inlet temperature to the initial treatment chamber and to intercool the juice between chambers as shown in Figures 6 and 7. The time for the liquid to travel from the chambers to the intercoolers ranged from 1.5 to 2 s. The temperatures of the juice immediately before and after the chambers were measured with 3.2 mm diameter chrome-constantan thermocouples (Omega Engineering, Inc., Stamford, CT). The temperatures were continuously logged to a data acquisition system (Dasytec USA, Amherst, NH, Dasylab version 5.0). The juice was

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quickly cooled after exiting the last chamber to less than 25°C using a stainless-steel heat exchanger (Madden Manufacturing, model SC0004). The time for the liquid to travel from the treatment chamber to the sample cooler ranged from 1.5 to 2 s.

Controls were performed to determine the effect of temperature alone. In order to ensure that the control liquid received the same time and temperature history as the treated liquid, the converged treatment chambers were replaced with ohmic heating chambers. These chambers consisted of stainless steel electrodes (Swagelok, Solon, OH, part no. SS-400-1-OR) inserted into 102 mm lengths of 6.4 mm ID plastic tubing. The ohmic heating chambers quickly brought the juice temperature up to the desired temperature. The control juice was identically held for 1.5 to 2 s before cooling.

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## Modeling of Radio Frequency Electric Fields

The anisotropic electric field strengths within the treatment chamber can be modeled with finite element analysis software such as QuickField™ (Tera Analysis Ltd, Svendborg, Denmark, version 5.0). Figure 8 presents the model's results for an electric field strength of 20 kV/cm within the converged section of the treatment chamber shown in Figure 5. The liquid flows through the electrode and enters a field-free region. It then flows into the central channel where the field is quickly raised to 20 kV/cm. The field within the channel is nearly uniform which ensures that all of the liquid is treated equally. The uniformity improves the energy efficiency of the process. By minimizing the regions within the treatment chamber where the electric field is too low to inactivate bacteria and only heats the liquid, approximately less than 5 kV/cm, the energy loss is minimized. Similarly, by minimizing the regions where the field is higher than needed to inactivate bacteria, the energy loss is minimized. Thus, the outlet temperature is lessened and the liquid is not overly treated.

## RFEF Nonthermal Inactivation of *E. Coli* in Orange Juice

The recently developed 80 kW RFEF pilot plant system successfully inactivated *Escherichia coli* K12 in pulp free orange juice at nonthermal conditions. The extent of microbial inactivation is dependent on the electric field strength, frequency and temperature.

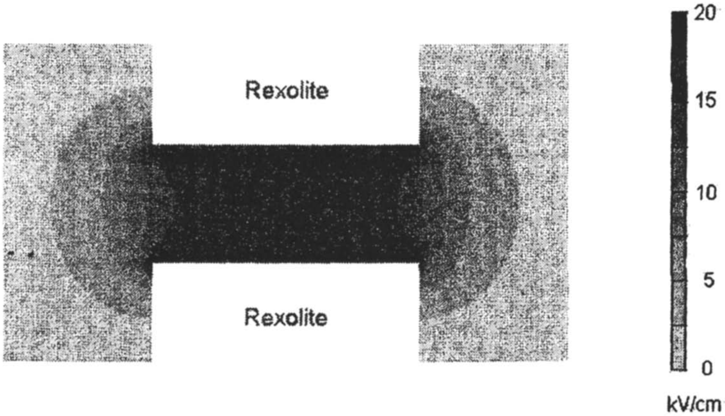

*Figure 8. Modeled anisotropic RFEF strength within the converged section of the treatment chamber shown in Figure 5.*

A series of experiments were performed at 21.1 kHz to determine the effects of electric field strength and temperature on inactivation. The RFEF process with two treatment chambers in series was used as shown in Figure 7. The treatment chambers used were the same as presented in Figure 5. The flow rate of orange juice was 1.4 l/min. The population of *E. coli* in orange juice was reduced by 3.2 log after being exposed to a 15 kV/cm peak electric field at a treatment time of 190 µs, inlet temperature of 40°C, outlet temperature of 65°C, and hold time of 2 s (Figure 9). Increasing the field strength to 20 kV/cm at the same temperature resulted in a reduction in *E. coli* of 3.9 log. When the juice was ohmicly heated at the same frequency, 21.1 kHz, to the same outlet temperature, 65°C, and held for the same time, 2 s, the population of *E. coli* was reduced by only 0.5 log. Therefore, RFEF processing reduced the population of *E. coli* in orange juice by 3.4 log relative to the control. The nonthermal inactivation is believed to be due to dielectric breakdown of the cells (*12*). Using the same RFEF pilot plant system, *E. coli* in apple juice was reduced by 2.1 log after being exposed to a 20 kV/cm peak electric field at a treatment time of 190 µs, outlet temperature of 65°C, and hold time of 2 s (*9*). The results of the present study successfully extended the RFEF process to inactivating *E. coli* in orange juice.

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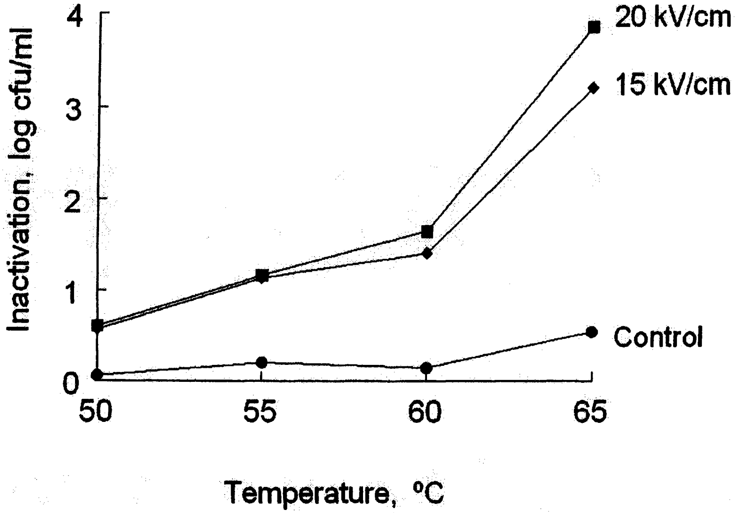

*Figure 9. Effects of temperature and electric field strength on the inactivation of E. coli at 190 μs RFEF treatment time and 2 s hold time (1.4 l/min flow rate). Means of two replicate experiments.*

Experiments were conducted to determine the effect of frequency on inactivation. The inactivation of *E. coli* in orange juice was substantially increased as the frequency was decreased from 40.1 kHz to 21.1 kHz as shown in Figure 10. Similar results were obtained in previous studies. Using a bench scale RFEF system, a significantly greater inactivation of *E. coli* in apple juice occurred at frequencies of 15 and 20 kHz compared to frequencies of 30 to 70 kHz (*8*). Using a pilot plant system, greater inactivation of *E. coli* in apple juice was observed as the frequency was decreased from 40.1 kHz to 21.1 kHz (*9*). These results are extremely interesting, not only because they indicate that the RFEF process could be more efficient at even lower frequencies, but also because RFEF equipment costs should be significantly less at lower frequencies as well.

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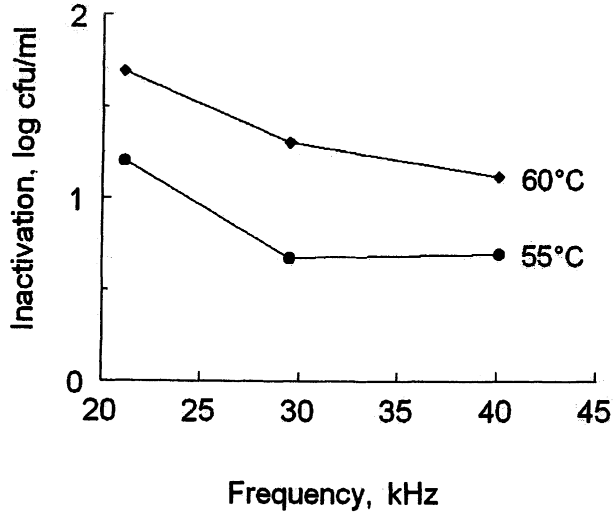

*Figure 10. Effect of frequency on the inactivation of E. coli at 20 kV/cm, 190 μs RFEF treatment time and 2 s hold time. Means of two replicate experiments.*

## Electrical Costs of RFEF Processing of Orange Juice

The energy costs of alternative pasteurization processes are an important factor in determining whether the new technologies will be commercialized. The electrical costs were estimated for the case of RFEF processing of orange juice at 15 kV/cm and 65°C. At these conditions, the population of *E. coli* was reduced by 3.2 log and the energy applied was approximately 120 J/ml. The estimated energy required for a 5 log reduction using pulsed electric fields (PEF) ranges from 100-400 J/ml (*13, 14*). It is probable that the RFEF electrical costs for a 5 log reduction will be similar to those of PEF as they are both considered electroporation processes (*8*). Based on the U.S. Department of Energy's data for the average industrial electric price for the year 2004 of $0.051/kWh, the energy cost for the RFEF process was approximately $0.0017 per liter of orange juice. For comparison, conventional thermal pasteurization, with heat regeneration or recovery, costs only $0.0005 per liter.

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## Nutrition and Quality of RFEF Processed Orange Juice

Two of the commonly occurring degradations in juice quality are non-enzymatic browning and loss of ascorbic acid. An experiment was conducted to ascertain the effect of RFEF processing on these two aspects of juice quality. Pulpfree orange juice was processed at 20 kV/cm and 65°C with a hold time of 2 s. At these conditions, the population of *E. coli* was reduced by 3.9 log. Samples of orange juice were taken before and after RFEF processing and were analyzed for browning and ascorbic acid.

Vitamin C (ascorbic acid) was measured using a HPLC method as described earlier (*15*). Orange juice was centrifuged at 12,000 g for 10 min at 5°C in a Sorvall RC2-B refrigerated centrifuge (Kendro Laboratory Products, Newtown, CT). The supernatant was filtered through a 0.45 μm Acrodisc LC 13 PVDF syringe filter (Gelman Sciences, Ann Arbor, MI) before being analyzed using a Hewlett Packard Ti-series 1050 HPLC system (Agilent Technologies, Palo Alto, CA). The HPLC system consists of an autosampler, an integral photodiode-array detector, an autoinjector and a Hewlett-Packard Rev. A02.05 Chemstation. Injection volume was 20 μl. Separation of compounds was achieved with an Aminex HPX-87H organic acids column (300 × 7.8 mm) fitted with a microguard cation H+ eluted with a mobile phase of 5 mM sulfuric acid at flow-rate of 0.5 ml/min. Column temperature was maintained at 30°C using a column heater (Bio-Rad Laboratories, Hercules, CA). Ascorbic acid was monitored at 245 nm and calculated from an ascorbic acid standard.

To measure browning, orange juice was centrifuged at 12,000 g for 10 min at 5°C (*16*). The absorbance of the supernatant at 420 nm was measured using a spectrophotometer (Shimadzu UV-1601 spectrophotometer, Shimadzu Scientific Instruments, Columbia, MD).

Many fruit and fruit juices are rich in ascorbic acid (Vitamin C). Ascorbic acid is, however, sensitive to many processing and storage conditions. It is known that exposure to high temperatures during pasteurization results in a considerable loss of ascorbic acid. For example, pasteurization (90°C for 60 s) of fresh orange juice resulted in a 2.4% loss in ascorbic acid (*17*). No measurable loss in ascorbic acid was observed due to RFEF process (data not shown), probably due to the low treatment temperature and duration. The errors of analysis were probably larger than the loss (if any) of ascorbic acid. Uemura and Isobe (*18*) used a 20 kHz RFEF apparatus to study inactivation of *Bacillus subtilis* spores in orange juice. The orange juice was RFEF processed at 121°C under pressurized conditions to elevate the boiling point. A 16.3 kV/cm field reduced the viable *B. subtilis* spores by 4 log in <1 s of treatment. Only 10% of the original ascorbic acid in the orange juice was destroyed after RFEF treatment. In our experiment, the juice was RFEF processed at 65°C, a temperature much lower than 121°C.

Non-enzymatic browning is due to Maillard-type reactions of sugars, amino acids and ascorbic acid. The reactions, influenced by many factors (such as

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temperature and oxygen), not only lead to browning and loss of ascorbic acid, but also produce compounds that contribute to off-flavor of juice. The oxidation of ascorbic acid can play an important role in the browning of fruit juice. No change in brownness of orange juice was observed as a result of RFEF treatment (data not shown), coinciding with the complete retention of ascorbic acid.

## RFEF Nonthermal Inactivation of *E. coli* in Apple Cider

*Escherichia coli* K12 in apple cider was successfully inactivated at nonthermal conditions using the 80 kW RFEF pilot plant system. The extent of microbial inactivation is dependent on the electric field strength and temperature.

A series of experiments were performed at 21.1 kHz to determine the effects of electric field strength and temperature on inactivation. The RFEF process with three treatment chambers in series was used as shown in Figure 6. The treatment chambers used were the same as presented in Figure 5, except that the diameter and length of the central channel were 1.4 mm and 2.3 mm, respectively, and the space between the end of each of the electrodes and the central channel was 2.0 mm. The flow rate of cider was 1.5 l/min. The population of *E. coli* in apple cider was reduced by 2.4 log after being exposed to a 20 kV/cm peak electric field at a treatment time of 140 μs per treatment chamber, outlet temperature of 55°C, and hold time of 2 s per treatment chamber (Figure 11). Increasing the temperature to 60°C at the same field strength and time resulted in a reduction in *E. coli* of 5.0 log. When the cider was ohmicly heated at the same frequency, 21.1 kHz, to the same outlet temperature, 60°C, and held for the same time, 2 s per ohmic treatment chamber, the population of *E. coli* was reduced by only 0.2 log. Therefore, RFEF processing reduced the population of *E. coli* in apple cider by 4.8 log relative to the control. Previously, *E. coli* in apple juice was reduced by 2.1 log after being exposed to a 20 kV/cm peak electric field at a treatment time of 140 μs per treatment chamber, outlet temperature of 65°C, and hold time of 2 s using a RFEF pilot plant system with two treatment chambers in series such as shown in Figure 7 (*9*). The better results obtained with cider are probably due to the fact that 3 treatment chambers were employed rather than two, and that the total treatment time was 120% longer. The results of the present study successfully extended the RFEF process to inactivating *E. coli* in apple cider that contains solids.

Experiments were conducted to determine the effect of frequency on inactivation. The inactivation of *E. coli* in apple cider was similar as the frequency was varied from 21.1 to 40.1 kHz. In the case of *E. coli* in orange juice, presented earlier in this chapter, inactivation improved as the frequency decreased. The variation in results may be due to the use of different numbers of treatment chambers and treatment times. The effect of frequency needs to be studied in greater detail because, if greater inactivation occurs at lower frequencies, the energy operating costs could be reduced. In addition, RFEF equipment costs may be significantly less at lower frequencies.

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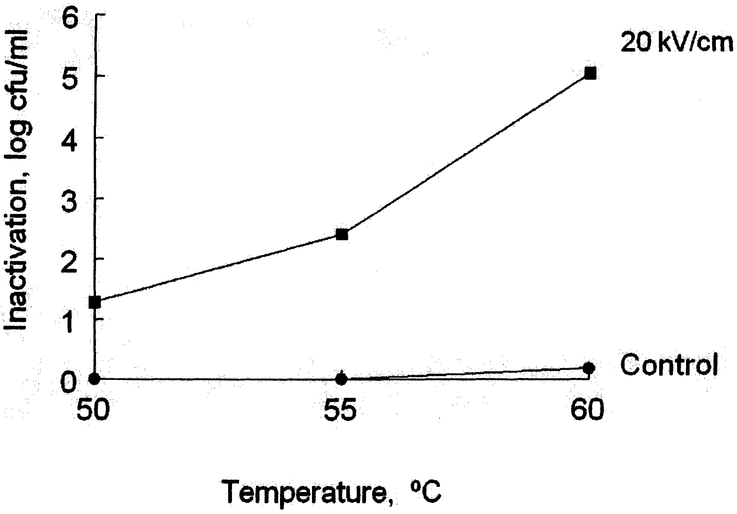

*Figure 11. Effects of temperature and RFEF on the inactivation of E. coli at the following condition: 3 treatment chambers, 140 μs treatment time per treatment chamber, and 2 s hold time per treatment chamber (1.5 l/min flow rate). Means of two replicate experiments.*

## Scanning Electron Microscope Imaging of *E. coli* Inactivated Using RFEF

Very little is known about the mechanism of inactivation by either PEF or RFEF. In order to investigate this phenomenon, scanning electron microscope (SEM) images were produced for *E. coli* that were thermally treated, nonthermally treated using RFEF, and untreated.

Deionized water was inoculated with *E. coli* culture to yield an 8.6 log cfu/ml bacterial cell suspension. This high population was necessary to generate SEM images containing multiple bacteria. Water was used instead of apple cider because particulates had been found to interfere with the imaging of the bacteria. Hydrochloric acid was mixed with the water, before adding the cultures, to reduce the pH to 4.4 which is typical of cider. The populations of the untreated cells were not affected by the pH reduction.

For the RFEF treatments, the bacterial cell suspension was processed using the same setup as the apple cider, except that only two chambers were used instead of three as shown in Figure 6. The processing conditions were 25 kV/cm and 55°C with a hold time of 2 s per chamber. To increase the inactivation, the product was recycled back to the feed tank. In all, the bacterial cell suspension was processed 24 times for a total hold time at 55°C of 1.6 min. The population of *E. coli* was reduced by 4.8 log.

For the thermal treatments, 10 ml of the bacterial cell suspension was placed in a test tube and submerged in a water bath. To get an inactivation equivalent to that obtained using RFEF processing, the required time and temperature were 75°C and 5 min. The population of *E. coli* was reduced by 5.4 log. For comparison to the RFEF processing, a sample was held at 55°C for 5 min and the population of *E. coli* was reduced by less than 0.1 log.

Aliquots (50 µL) of bacterial cell suspensions were deposited onto 10 mm dia. glass coverslips. After ~ 30 s, the coverslips were gently immersed into 2 ml volumes of fixative solution, 2.5% glutaraldehyde-0.1M imidazole buffered at pH 7.0, in a multi-well plate. After 2 h at room temperature, the plate was sealed and stored at 40°C. In preparation for scanning electron microscopy, the fixative solution was removed and replaced with several ~2 ml aliquots of imidazole buffer to remove glutaraldehyde and bacterial cells on the coverslips were dehydrated by sequential immersion of the coverslips in aliquots of graded solutions of ethanol (50%, 80% and absolute) before critical point drying from liquid $CO_2$. The dried coverslips were glued to specimen stubs and coated with a thin layer of gold by DC sputtering in a ScanCoat 6 sputter coater (BOC Edwards, Wilminton, MA). Samples were viewed and digital images were collected using a Quanta 200 FEG scanning electron microscope (FEI Co., Inc., Hillsboro, OR) in the high vacuum, secondary electron imaging mode of operation.

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Secondary electron images of the bacterial cells adhering to the glass coverslips after experimental and preparative treatments are illustrated in Figure 12. Control cells were typically individual, rod-shaped with smooth surfaces. But both groups of treated (thermal and nonthermal) cells were mostly loosely clumped into small groups containing three to ten cells with various superficial irregularities of the surface of nearly every cell. The shapes of thermally-treated cells were distorted by large, irregular depressions and evaginations of their surfaces. The shapes of nonthermally treated cells were less distorted than those thermal treated, but the visible surfaces had at least a few attached small vesicles.

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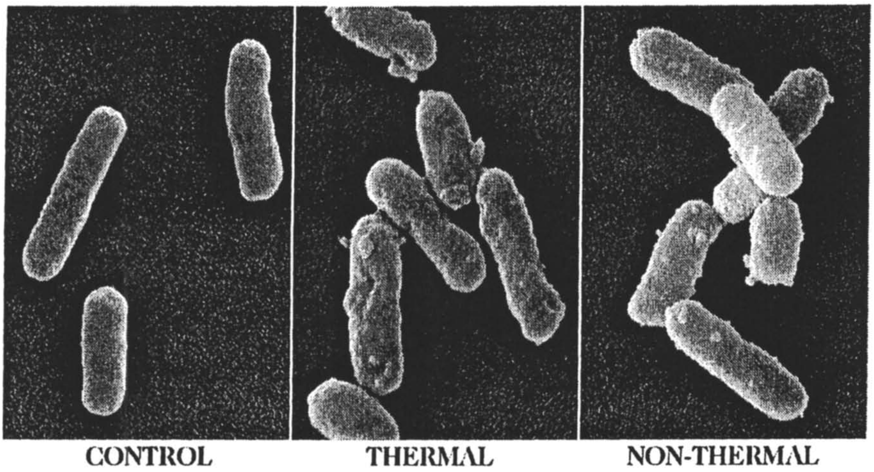

*Figure 12. Scanning electron microscope images of untreated, thermally treated (at 75 °C for 5 min), and nonthermally treated E. coli using RFEF. The thermal and nonthermal inactivations were 5.4 and 4.8 log, respectively.*

The radio frequency electric fields (RFEF) process has been shown to reduce the population of *Escherischia coli* in orange juice at 50°C. Inactivation is dependent upon the electric field strength and temperature. Better inactivation has been observed at radio frequencies near 20 kHz as compared to frequencies near 40 kHz. The calculated electrical cost is $0.0017 per liter of orange juice. There is no change in brownness of orange juice as a result of RFEF treatment, nor is there any loss of ascorbic acid. The RFEF process has also been shown to reduce the population of *E. coli* in apple cider at 50°C. Inactivation is dependent upon temperature, but, in this case, is independent of frequency. The difference in results may be due to the use of slightly different RFEF systems. Scanning electron microscope images of untreated, thermally treated, and RFEF nonthermally treated *E. coli* showed noteworthy differences indicating that the mechanisms for thermal and RFEF inactivation are dissimilar.

Although remarkable progress has recently been made in the development of the nonthermal RFEF process, more research needs to be done before it can be commercialized. The RFEF process needs to be further scaled up to be of commercial interest. Additional quality and cost analyses must be performed. The stability of the equipment, including the metal electrodes, at longer operational times must be studied. Finally, RFEF processing at lower frequencies, where the efficiency may be enhanced, deserves attention.

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## Chapter 10

# High Hydrostatic Pressure Processing

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This paper overviews the nonthermal food processing technology of high hydrostatic pressure processing. In brief segments, the fundamental chemistry of hydrostatic pressure applications, generation of adiabatic heat, and historical perspective of the pressure processing of foods are presented. The key process parameters of the technology are discussed with regard to some current commercial products. The most substantial portion of the article deals with the response of microorganisms to the process, focusing primarily on problematic varieties involved in spoilage and foodborne illness with specific examples highlighted among vegetative bacteria, bacterial endospores, human infectious viruses, animal viruses, fungi, protozoa and parasites. The paper concludes with a section presenting examples of hurdle technology incorporating high pressure processing in food products. Additional process factors that are presented in combination with pressure include temperature, bacteriocins, modified-atmosphere packaging, preservative enzymes, and use of pulsed electric fields.

According to Le Chatelier's principle, pressure enhances reactions leading to volume reductions, whereas processes involving a volume increase are inhibited by pressure application (*1*). This principle governs the structural rearrangements that take place for proteins upon pressurization. At ambient temperature high pressure usually disrupts relatively weak chemical bonds such as hydrogen bonds, hydrophobic bonds, and ionic bonds. In contrast, covalent bonds remain unaffected so primary structure remains intact during and after pressurization. In general, high pressure denatures proteins, solidifies lipids and destabilizes biomembranes. It is this destabilization or leakage of membranes that is the primary mechanism in which vegetative microorganisms are inactivated.

Applications of high hydrostatic pressure induce the generation of heat from compressed fluids. The heat of compression is also called adiabatic heating. The temperature increase during compression under adiabatic conditions can be described by the following equation (*2*):

$$\frac{dT}{dP}=\frac{\alpha T}{\rho C_p}$$

where $T$ = temperature (K), $P$ = pressure (Pa), $\alpha$ = thermal expansion (1/K), $\rho$ = density (kg/m$^3$), $Cp$ = heat capacity (J/kg•K). This equation indicates that relatively high initial temperature can lead to a relatively large temperature increase rate (°C/MPa). For example, temperature increases of water due to compression heating are 2.8, 3.8, and 4.4°C/100 MPa at initial temperatures of 20, 60, and 80°C (*3*).

Bert Hite was the first to use high hydrostatic pressure processing (HPP) as a food preservation method. He pressure-processed a variety of foods and beverages in the late 1890s and early years of the 20th Century (*4, 5*). Since those initial efforts, occasional attempts by others were made through the century to study the pressure treatment of foods, but it was not until the early 1980s that the potential of HPP as a food process came to be realized. With improvements in the technology and design of pressure-generating equipment, HPP research resumed in the U.S. and Japan and proliferated elsewhere. With continued demand for minimally processed, high-quality foods, HPP emerged as a very promising method to reliably deliver safe foods that lacked the undesirable changes to sensory quality and nutrient content so often characteristic of foods receiving excessive thermal treatments.

In Japan, pressure-treated jams and jellies were the first commercialized food products that employed pressure for preservation. These fruit products were initially marketed in 1991 and they continue to be sold in Japan with the addition

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of salad dressings and a wide range of fruit juices. In 2001, pressure-treated guacamole entered the U.S. marketplace, followed by HPP salsa. Pressure-processed chopped onions are anticipated for sale as an ingredient in premium salad dressings in 2004. Also anticipated in 2004 are applesauce and applesauce/fruit blends packaged as eat-on-the-go single-serve flexible tubes from a Canadian venture and fruit "smoothie" products from Mexico for North American distribution, respectively.

## Process Parameters

Most pressure units used to process foods or food ingredients generate pressures in the range between 100 and 800 MPa. A pressure of 580 MPa (85,000 psi) has been used to commercially process guacamole. Of course, the shortest length of time at pressure as possible is preferred; foods are exposed to the set pressure point from milliseconds to over 20 min, although times of 5 to 7 min are usually more common (*6, 7*). The product temperature during pressurization can be controlled and maintained below 0°C or above 100°C; however, current industrial units normally use ambient temperatures. HPP systems can be used semi-continuously for pumpable fluid foods or, as is usually the case, in a batch manner for pre-packaged solid or semi-solid foods.

The major critical process factors for HPP include treatment pressure, holding time at pressure, come-up time to achieve pressure, decompression time, initial temperature of food materials, process temperature, temperature distribution in the pressure vessel as a result of adiabatic heating, characteristics of the product (*e.g.*, pH, composition and water activity), the packaging material and types of microorganisms found in the foods (*8*). As long as food packages fit into the treatment chamber, package size and shape are not critical factors because pressure acts instantaneously and uniformly throughout the chamber and food mass. There is no pressure gradient in the food. If pressure pulsing is used, additional process factors include pulse shape (*i.e.*, the waveform), frequency and pulse-pressure magnitudes.

## Response of Microorganisms to Hydrostatic Pressure

The key value for use of hydrostatic pressure in food processing is the inactivation of microorganisms contained in the food. A few microbial life forms, such as bacterial endospores and viruses (*i.e.*, poliovirus) are unaffected by pressure alone and require pressure treatment at elevated temperatures or some other action to realize a feasible level of inactivation; however, for most other microorganisms of concern in foods pressure will usually deliver a level of

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inactivation that has sometimes been referred to as "pasteurization". Most types of detrimental food microorganisms, *e.g.*, vegetative bacteria, most human infectious viruses, fungi, protozoa and parasites, can be considerably reduced or eliminated by exposures to high pressure. The yardstick for microbial inactivation of HPP usually starts with the approximation that gram-negative bacteria are usually more sensitive to pressure inactivation than gram-positive vegetative bacteria and then proceeds up the evolutionary ladder in biology to note that fungi are more susceptible to pressure inactivation than bacteria while protozoa and parasites are more sensitive than fungi, and the higher the organisms on the ladder, the greater the sensitivity to pressure (*6*). Viruses are notable in that a broad range of sensitivities (or from another point-of-view, resistances) is evident from studies that have been done.

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As might be expected with any generalization, exceptions to the rule are not uncommon. A range of pressure sensitivities are found within most microbial groups. For example, most fungal conidiospores and ascospores can usually be inactivated at pressures between 300-450 MPa at ambient temperature (*9, 10, 11, 12, 13*), but in a study on dormant ascospores of *Talaromyces macrosporus*, treatment over a pressure range of 200 to 500 MPa and 20°C activated dormant ascospores and caused little or no inactivation of the fungi. Higher pressures of 500 to 700 MPa (20°C) were required to inactivate the ascospores; however, application of 700 MPa for 60 min only reduced the spore population by less than 2 $\log_{10}$ units, indicating the high resistance of some ascospores to pressure (*14*). Some general examples or common responses of vegetative bacteria to inactivation by pressure include more than a 5- $\log_{10}$ reduction in viable *Staphylococcus aureus* counts after pressure treatment of 600 MPa for 15 min at 20°C in ultra-high temperature (UHT) milk (*15*), and a 6-$\log_{10}$ inactivation of a pressure-resistant strain of *Escherichia coli* O157:H7 (NCTC 12079) after exposure to 550 MPa for 5 min and 20°C in orange juice over the pH range 3.4 to 5.0 (*16*).

Bacterial endospores are the most difficult life-forms to eliminate with hydrostatic pressure. Application of pressure alone will not inactivate bacterial endospores. In 1932, Bassett and Macheboeuf (*17*) very capably demonstrated this fact by detecting viable spores of *Bacillus* after a 45-min exposure to >1,724 MPa (250,000 psi) at ambient temperature. Thus, a hurdle approach that utilizes pressure in combination with other processes or factors is required to inactivate spores (*18*). Usually pressure treatment with mild heat (*e.g.*, 40 to 55°C) is used for substantial reduction of spore levels (*18, 19*). Presently, successful commercial preservation of foods utilizing HPP largely incorporate refrigerated storage of the product or a product pH below 4.5 in order to prevent the germination of spores of *C. botulinum* and other sporeforming bacteria. Production of commercially sterile low-acid foods employing HPP must overcome the high degree of resistance by bacterial spores.

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A phenomenon observed with other food processing methods is the protection food offers to microorganisms. As demonstrated in many laboratories, most foods are more protective to microorganisms when compared to inactivation of microorganisms in water, buffer or microbiological media. This is also true in pressure processing (*20, 21*). For example, Chen and Hoover (*22*) compared the resistance of *Y. enterocolitica* to high pressure in ultra-high temperature (UHT) milk and sodium phosphate buffer (0.1 M, pH 7.0). In buffer, pressurization of *Y. enterocolitica* at 350 MPa for 26 min, at 400 MPa for 11 min, and 450 MPa for 7.5 min reduced the counts of *Y. enterocolitica* by more than 8 $\log_{10}$ CFU/mL, while in milk these same processing conditions only reduced *Y. enterocolitica* counts in milk by less than 2.5 $\log_{10}$ CFU/mL.

Giddings and his coworkers (*23*) first examined the sensitivity of viruses to pressures and reported that inactivation of tobacco mosaic virus (TMV) required pressures as high as 920 MPa. From more recent investigations it now appears that most human viruses are substantially less resistant to pressure than TMV. Most of viruses of food safety concern can be inactivated at pressures of 450 MPa or less.

An exposure to pressures between 400 to 600 MPa for 10 min eliminates $10^4$ to $10^5$ viable particles of human immunodeficiency virus (*24*). Certain viruses can be inactivated at even lower pressure magnitudes. Jurkiewicz *et al.* (*25*) showed that pressurization of simian immunodeficiency virus (SIV) at 250 MPa for 1 h at 21.5°C reduced its infectivity by 5-$\log_{10}$ units, while pressurization at 200 MPa for 3 h or 150 MPa for approximately 10 h was needed to obtain the same level of destruction. A 10-min exposure to 400 MPa eliminates 8-$\log_{10}$ plaque-forming unit (PFU) population of herpes simplex virus type 1 (HSV-1), and a 10-min exposure to 300 MPa inactivates 5-log $\text{PFU}_{10}$ populations of human cytomegalovirus (HCMV; *26*). A 10-min exposure to 400 MPa was shown to eliminate 5.5-$\log_{10}$ tissue culture infectious dose of HIV type 1 at 25°C (*27*). It appears that the sensitivities of viruses to pressure are not correlated to genetically related taxonomic groups or even between strains as from the same group.

Kingsley *et al.* (*28*) investigated pressure inactivation of viruses that contaminate raw shellfish. Five-min treatments at 275 MPa completely inactivated 7-$\log_{10}$ tissue culture infectious doses of feline calicivirus, a surrogate for norovirus. Five-min exposures to >450 MPa reduced 7 $\log_{10}$ PFU/ml of hepatitis A virus (HAV) in tissue culture medium to nondetectable levels. Interestingly, it was found that suspension of hepatitis A in seawater increased the pressure resistance of the virus as compared to treatment in culture medium. Five-min treatments at 600 MPa had no effect on poliovirus, which agreed with the work of Wilkinson *et al.* (*29*).

Apparently low temperature treatment at pressure promotes inactivation of viruses due to enhanced dissociation and denaturation of viral proteins (*30, 31*).

The explanation for this phenomenon is the specific and strongly temperature-dependent interaction of protein nonpolar groups with water. Low temperature under pressure promotes interaction of non-polar side chains to water decreasing the hydrophobic effect resulting in cold denaturation of proteins. Non-polar interactions are more affected by pressure because they are more compressible, which results in an additive effect of high pressure and low temperature that reduces the entropy of the system (*32*).

Oliveira *et al.* (*33*) examined the combined effect of pressure and low temperature on the stability of foot-and-mouth disease virus (FMDV), an animal virus that is of great concern to the meat industry. FMDV was found to be sensitive to pressure, pressurization at 240 MPa for 2 h caused a reduction of infectivity of 4-$\log_{10}$ units at room temperature and 6-$\log_{10}$ units at -15°C.

Exposure to 550 MPa for 30 sec inactivated *Cryptosporidium parvum* oocysts suspended in apple and orange juices by at least 3.4 $\log_{10}$, and 60-sec treatments efficiently rendered the oocysts nonviable and noninfectious (*34*). An exposure to 200 MPa for 10 min completely inactivated all *Anisakis* larvae isolated from fish tissues and suspended either in distilled water or in a physiological isotonic solution between 0 and 15°C. All larvae were killed when exposed to 140 MPa for 1 h (*35*). *Anisakis simplex* larvae inoculated into king salmon and arrowtooth flounder fillets were completely killed by treatments of 414 MPa for 0.5-1 min, 276 MPa for 1.5-3 min, and 207 MPa for 3 min (*36*); however, application of HPP to raw fish fillets was of limited success because of the significant whitening of the flesh caused by pressure treatment.

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## Examples of Microbial Inactivation in Food Products

Studies to investigate the use of HPP on fruit juices has been extensive. In early product development work, pressures of 200 MPa effectively killed yeasts and molds in freshly squeezed orange juice at ambient temperature (*9*). Neither freshly squeezed orange juice nor juice inoculated with yeasts and molds showed an increase in total counts after 17 months of storage at 4°C following a 400-MPa pressure treatment at 23°C (*10*). When apple, orange, pineapple, cranberry and grape juices were inoculated with ascospores and vegetative cells of *Zygosaccharomyces bailii* and pressurized at 300 MPa for 5 min, the populations of vegetative cells and ascospores were reduced by almost 5-$\log_{10}$ units and 0.5-1 $\log_{10}$ units, respectively (*13*); the ascospores proving more difficult to eliminate.

Significant variations in bacterial pressure resistance were demonstrated for different types of fruit juices. For example, a three-strain cocktail of *E. coli* O157:H7 was found to be most sensitive to pressure in grapefruit juice (8.3-$\log_{10}$ reductions) and least sensitive in apple juice (0.4-$\log_{10}$ reductions) when pressurized at 615 MPa (2 min and 15°C; *37*). The obvious difference in pressure resistance is unclear.

Some fruits or vegetables were examined for the potential of HPP treatment. A pressure of 340 MPa and 15 min extended the shelf-life of fresh-cut pineapple (*11*). Pressures of 300 and 350 MPa reduced the populations of gram-negative bacteria, yeasts and molds by at least one $\log_{10}$ in lettuces and tomatoes; however, the tomato skins loosened and peeled away, and lettuce browned (*12*).

The potential of HPP to reduce the microbial loads of certain seeds were also investigated. Garden cress, sesame, radish, and mustard seeds were immersed in water and exposed to different levels of pressures (250, 300, 350, and 400 MPa) at 20°C for 15 min (*38*). Seed germination on water agar was recorded up to 11 days after HPP. Radish and garden cress seeds were the most pressure-sensitive and pressure-resistant types, respectively. For example, after a 250-MPa treatment, radish seeds displayed 100% germination nine days later than untreated controls, while garden cress seeds attained 100% germination one day after the controls. Garden cress seeds were inoculated with suspensions of seven different kinds of bacteria (starting inocula $10^7$ CFU/g). Treatment at 300 MPa for 15 min and 20°C resulted in 6-$\log_{10}$ reductions of *Salmonella* Typhimurium, *E. coli* MG1655, and *Listeria innocua*, > 4-$\log_{10}$ reductions of *Shigella flexneri* and the pressure-resistant strain *E. coli* LMM1010, and a 2-$\log_{10}$ reduction of *Staphylococcus aureus*, but *Enterococcus faecalis* was not inactivated to a significant extent.

The effect of pressure processing on microorganisms in mechanically recovered poultry meat was investigated by Yuste and coworkers (*39, 40*)). Aerobic mesophiles in the meats were susceptible to HPP. Addition of nisin and meat acidification significantly enhanced pressure inactivation of both mesophilic and psychrotrophic microorganisms. Treatment of freshly ground raw chicken at 408, 616, and 888 MPa for 10 min resulted in microbiological shelf-lives of 27, 70, and >98 days, respectively, when the pressured samples were stored at 4°C; unprocessed chicken samples had a microbiological shelf-life of 3 to 4 days (*41*).

## Examples of HPP Combined with Other Approaches

It is frequently observed that high pressure, in combination with other preservation factors, enhances bacterial inactivation and results in longer shelf-life of treated foods. Since capital costs of high pressure equipment increase exponentially with operating pressures, process costs are related to operating pressures (*8*). Therefore it is economically beneficial to use lower levels of pressure in combination with other processing techniques in order to obtain the

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desired target levels of microbial inactivation while maintaining a maximum degree of sensory and nutrient qualities for the product.

It is well-established that elevated temperatures promotes pressure inactivation of microorganisms. Chen and Hoover (*42*) found that a 5-min treatment of 500 MPa at 50°C resulted in a more than 8-$\log_{10}$ reduction of *L. monocytogenes* in milk, while at 22°C a 35-min treatment was needed to obtain the same level of inactivation. Patterson and Kilpatrick (*15*) found that simultaneous application of high pressure and mild heat was more lethal to *E. coli* O157:H7 and *S. aureus* than either treatment alone. A 5-min treatment of 500 MPa at 50°C resulted in a 6.0-$\log_{10}$ reduction of *S. aureus* in UHT milk, while a <1.0-$\log_{10}$ reduction in numbers was achieved with either treatment alone. Ponce *et al.* (*43, 44*) found that 50°C was most effective in the pressure inactivation of the two gram-negative bacteria, *Salmonella* Enteritidis and *E. coli*, when compared to pressure treatments at -15, 2, and 20°C. Carlez *et al.* (*45*) also found that elevated temperature enhanced the destruction of *Citrobacter freundii, Pseudomonas fluorescens* and *Listeria innocua* in minced beef muscle. Again, 50°C was the most effective treatment temperature for inactivation when compared to treatments at 4, 20 and 35°C.

Use of the bacteriocin, nisin, has been shown to not only increase pressure inactivation of gram-positive bacteria, but also increase inactivation of gram-negative bacteria that are usually insensitive to nisin. Enhanced inactivation of gram-negative pathogens, such as *E. coli* O157:H7, *S.* Enteritidis, *Salmonella* Typhimurium, and *Shigella sonnei,* have been demonstrated when nisin is used in HPP (*46, 47, 48*). Nisin also increases pressure inactivation of spores of *Bacillus coagulans, B. subtilis* and *C. sporogenes* (*18, 49*). Similar synergy with HPP has been shown for the bacteriocins, pediocin AcH (*50*) and lacticin 3147 (*51*).

Amanatidou *et al.* (*52*) studied the potential of using high pressure in combination of MAP for the preservation of salmon. Treatment at 150 MPa for 10 min at 5°C extended the shelf-life of salmon by 2 days compared to untreated, vacuum-packed salmon. MAP storage (50% $O_2$+50% $CO_2$) alone extended the shelf-life of salmon for 4 days. When salmon was subjected to the combined treatment of high pressure and MAP, the threshold value for microbial spoilage of salmon (7.0-7.2 $\log_{10}$ CFU/g) was not reached for at least 18 days at 5°C. Spoilage microorganisms (lactic acid bacteria, *Shewanella putrefaciens*) and pathogens (*L. monocytogenes* Scott A, *S.* Typhimurium) inoculated on salmon were more susceptible to high pressure in the presence of MAP. Although bacterial growth on salmon was retarded, the combined high pressure processing and MAP treatments promoted a detrimental effect on color and changes in the balance of oxidative rancidity.

Lysozyme will enhance pressure inactivation of some gram-negative bacteria. Masschalck *et al.* (*53*) studied the inactivation of six different

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gram-negative bacteria (*E. coli*, *P. fluorescens*, *S.* Typhimurium, *S.* Enteritidis, *S. sonnei*, and *Shigella flexneri*) by high hydrostatic pressure treatment in the presence of hen egg-white lysozyme. The lysozyme increased the sensitivity of all the examined bacteria to pressure except for both *Salmonella* serotypes. Treatment at 300 MPa reduced the number of *E. coli* by 2.6 log CFU/mL in the presence of 100 μg of lysozyme/mL; when no lysozyme was added the reduction was 1.6 log CFU/mL.

The effect of PEF on the pressure inactivation of vegetative *B. subtilis* cells was studied by Heinz and Knorr (*54*). Simultaneous application of 200 MPa and PEF for less than 1 min did not result in any greater inactivation as compared to PEF treatment at atmospheric pressure. A synergistic effect for inactivation of *B. subtilis* was only observed when the pressure treatment time was extended to 10 min prior to the pulsed electric field treatment. If no PEF pulses was applied following a 30-min pressure application at 200 MPa, there was no significant reduction in plate counts. Such data suggest that a combination of these two processing technologies does not appear to be industrially worthwhile at the present time.

Conventional thermal processing utilizes heat to inactivate both microorganisms and enzymes in order to extend the shelf-life of the products and impart other qualities to the food. One should be mindful when comparing the inactivation kinetics of thermal processing to those generated by nonthermal processing methods because the cellular mechanisms of microbial inactivation differ so substantially; there are different rates of inactivation as well as different degrees of effectiveness in different food products. For HPP, as well as any other nonthermal food process currently under development, a broad knowledge base is essential in order to assist in the validation of the process for the marketplace.

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## Chapter 11

# Microbiological and Safety Aspects of Pulsed Electric Field Technology

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Consumers are increasingly aware of the health benefits and risks associated with consumption of food. Consumers also perceive fresh food as healthier than the heat-treated; therefore, the industry is now seeking alternative technologies to maintain most of the fresh attributes, safety and storage stability of food. Pulsed electric field (PEF) is one of these promising alternative technologies. Satisfactory evaluation of a new preservation technology, such as PEF, depends on reliable estimation of its efficacy against pathogenic and spoilage food-borne microorganisms. Research on alternative technologies was initially focused on process design, product characteristics and kinetics of microbial inactivation. The success of these new technologies, however, depends on the progress in understanding microbial physiology and behavior of microbial cell during and after the treatment. Consequently, this presentation reviews the PEF technology with emphasis on (i) mechanisms of microbial inactivation, (ii) patterns of inactivation kinetics, and (iii) microbial resistance mechanisms.

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Interest in the development of new food processing technologies has increased dramatically over the past two decades. This interest has been driven by consumer demand for food with fresh-like taste, crisp texture and natural color. Consumers are also increasingly becoming aware of food-borne disease hazards and are concerned about the safety of their food supply. Developments in nonthermal processing technologies have been advanced by both industry and academia in an attempt to meet the challenge of producing safe processed food of a high quality. There is no doubt that high quality food can be produced through the use of non-thermal processing technologies. The safety and microbiological quality of food processed using these technologies, however, needs to be affirmed. The safety of foods processed using pulsed electric field technology will be addressed in this chapter.

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## New Technologies and New Safety Strategies

Food is deemed unsafe if it constitutes either a physical, chemical or biological hazard to the consumer. Physical hazards may result, for example, from the presence of pieces of metal or glass in foods. Physical hazards are unlikely to increase when traditional technologies are substituted by novel technologies. Chemical hazards occur when deleterious substances occur naturally within the food, or are either intentionally or accidentally added to it. Hazardous chemicals (e.g. nitrosamine produced during the curing of meat) may also be produced during food processing. Information pertinent to the potential development of hazardous chemicals during food processing by non-thermal technologies is currently lacking. Chemical hazards associated with these new technologies will not therefore be addressed in this Chapter. Biological hazards are associated with the presence of pathogens, i.e. viruses, bacteria, fungi and parasites (Table I) that cause food-transmitted diseases. Food safety is currently compromised more often by biological than by physical or chemical agents. Non-thermal food processing technologies therefore target maximum impact against such biological hazards.

Food processors currently rely on a variety of methods for food preservation. These conventional methods include heating, dehydration, freezing, and the addition of preservative ingredients. Heat is the most commonly used preservation factor and heat-treated food generally has a good safety record. When properly applied, heat can eliminate bacteria, fungi, viruses, parasites, and enzymes, which are the biological agents that cause spoilage or compromise food safety. The dosage of conventional preservation factors can be varied to accomplish microbial inactivation over a broad spectrum. For example, when heat is applied to milk at 71.6°C for 15 seconds, a 5-log kill, at least, of non-spore forming bacterial pathogens occurs, and the resulting product is considered pasteurized. However, heating milk at 145°C for a few seconds produces a commercially sterile Ultra High Temperature (UHT)-treated product. This UHT treatment is presumed to be a 12-D process when targeting *Clostridium botulinum* spores.

**Table I. Microorganisms Causing Foodborne Diseases**

| *Gram-Negative Bacteria* | *Gram-Positive Bacteria* | *Molds* | *Parasites* |
|---|---|---|---|
| *Escherichia coli* | *Listeria monocytogenes* | *Aspergillus* | *Cryptosporidium* |
| *Salmonella* | *Staphylococcus aureus* | *Penicillium* | *Giardia* |
| *Shigella* | *Bacillus cereus* | *Fusarium* | *Entamoeba* |
| *Yersinia* | *Clostridium botulinum* | *Clavicepts* | *Toxoplasma* |
| *Campylobacter* | *Clostridium perfringens* | | *Fasciola* |
| *Vibrio* | | | *Trichinella* |
| *Aeromonas hydrophila* | | | |

Nonthermal technologies have been advanced to replace conventional heat treatments. Pulsed electric field (PEF) has been developed as a nonthermal food preservation method to inactivate microorganisms without significant loss in the flavor, color, taste and nutrients of foods (*1*, *2*). PEF treatment uses pulses of high intensity electric field generated between two electrodes. PEF punctures cell membrane of microorganisms to achieve inactivation. PEF treatment is attained as nonthermal by the use of a very short pulse duration time in microseconds. High pressure processing (HPP), on the other hand, relies on an extremely high pressure in hundreds of mega Pascals to denature the membrane proteins of microorganisms. These new technologies cannot however achieve the broad microbial lethalities that are currently attainable by heat treatment. Presently PEF and HPP technologies can accomplish the equivalent of pasteurization when applied at lethal doses. The achievement of commercial sterility may be feasible when nonthermal and conventional technologies are combined.

The application of nonthermal technologies to foods is more likely to result in stress or injury rather than to cause the death of microorganisms. An abundance of injured microbial cells in non-thermally-processed food may create new challenges to food processors and regulatory agencies. The detection of low levels of pathogens in food is a difficult task particularly when cells are injured. Safety of the nonthermally processed product is compromised if food and storage conditions favor recovery of injured cells. Stress of pathogens by non-thermal technologies is a concern and the adaptation of cells to such stress may

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constitute a microbial hazard. Non-thermal technologies therefore introduce new challenges, and thus warrant the implementation of new safety strategies.

## Kinetics of Microbial Inactivation

The inactivation of microorganisms during food processing either by conventional or novel technologies, is dependent on (a) processing variables, (b) properties of the treated food and (c) characteristics of the treated microorganism. This Section will review the dependence of microbial inactivation on processing variables and will emphasize the contribution of these factors to inactivation kinetics.

Thermal and nonthermal preservation technologies cause microbial inactivation in a dosage-dependent fashion. Where heat treatment is applied, for example, temperature and hold-time define the thermal process, i.e. the higher the temperature and the longer the hold-time, the greater the microbial lethality. Dependence of microbial inactivation, at a given temperature, on treatment time follows a pattern similar to that of chemical first-order reaction kinetics. Linearity of semi-log survivor plots makes it possible to measure inactivation rate parameters and allow for reasonable predictability of the treatment process. Data from survivor plots are commonly used to measure the decimal reduction time (D-value) using the following formula:

$$D-value=\frac{-t}{Log(N_t/N_0)} \qquad (1)$$

where $N_t$ is the count of survivors at time t, and $N_0$ is the initial count at time 0.

The D-value is an important parameter which describes thermal inactivation kinetics at a given temperature. In practical terms, D-value is equivalent to the treatment time required to decrease the number of the treated microorganisms by one log cycle, i.e. 90%. A semi-logarithmic plot of D-values vs. temperature (T) allows for measurement of a thermal resistance parameter or z-value, which is calculated as follows:

$$z-Value=\frac{T_2-T_1}{Log(D_1/D_2)} \qquad (2)$$

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where z-value represents the change in temperature required to cause a ten-fold alteration in the D-value. A concise review of kinetic models for fitting microbial inactivation data is provided by Xiong et al. (*3*).

The relationship between microbial inactivation and nonthermal treatment dosage is however more complex. Several interrelated processing variables (critical process factors) in nonthermal technologies require closer monitoring than in case of heating.

Inactivation of microorganisms by PEF is also dependent on several processing variables, food properties and characteristics of the treated microorganism. Processing variables of greatest significance (i.e., critical process factors) include electric field intensity (E), treatment time, treatment temperature, and pulse wave shape (*4*). Treatment time is the product of the number of pulses received by the food and the pulse duration. Exponentially decaying, square-wave and bipolar pulse wave shapes are commonly used in experimental PEF systems. In general, the efficacy of PEF against microorganisms increases proportionally to the electric field intensity, total treatment time, and treatment temperature and with a square pulse wave.

It is generally agreed that the PEF process can be reasonably defined by the electric field strength and total treatment time. Monitoring these two critical process parameters allows a reasonably good prediction of microbial inactivation (*5*). Inactivation kinetics for PEF may therefore be simplified by plotting counts of survivors at a given electric field strength and the corresponding treatment times. For a linear survivor plot, the D-value at the tested electric field strength can be calculated as indicated earlier for thermal treatments. Measured D-values can then be described as a function of electric field strength using a dose-response model similar to that applied for heat treatment (1). This first order kinetics, however, does not apply to the majority of experimental data. Nonlinear data led investigators to search for alternative models which better describe the kinetics of microbial inactivation during PEF processing. Hülsheger et al. (*5*) applied a kinetic model that correlates the fraction of survivors ($N/N_0$) with electric field strength (E), and treatment time (t) as follows:

$$N / N_0 = (t / t_c)^{\{-(E-E_c)/k\}} \tag{3}$$

Where $t_c$ is a critical treatment time, $E_c$ is a critical field strength and k is a constant.

More recently, Peleg (*6*, *7*) applied another kinetic model to sigmoid microbial inactivation curves resulting from PEF treatment. The model describes the ratio of survivors ($N/N_0$) as a function of the electric field strength (E) as follows:

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$$N / N = \frac{1}{1 + e^{(E - E_d)/k'}} \tag{4}$$

where k is related to the slope of the steep segment of the dose-response plot, and $E_d$ is a critical electric field value.

## Mechanisms of Microbial Inactivation

Microorganisms are affected to various degrees by processes that cause structural or functional damage to the cell. Results of this functional or structural damage include (a) inhibition of growth, (b) loss of the ability of cells to multiply (loss of viability), or (c) cell death (inactivation). The most common types of structural damage affect both the cell wall and the cytoplasmic membrane, leading to cell injury in mild treatments or cell lysis in case of severe processing. Microorganisms also are inactivated when subjected to processes that impair the functions of enzymes, DNA, ribosomes, or other essential constituents of the cell.

Sale and Hamilton (*8*, *9*) and Hamilton and Sale (*10*) conducted systematic studies on the effect of pulsed electric fields on the inactivation of microorganisms. According to these workers, intense electric pulses cause either permanent or temporary loss of the integrity of the microbial cell membranes. Their calculations showed that a minimum potential difference of 1 V across the membrane of the microorganism was required for loss of function as a semipermeable barrier between the cell and its environment.

A widely accepted mechanism for cell inactivation by PEF is based on the concept of the electrical breakdown of the cell membrane (*11*, *12*). The cell membrane can be modeled as a capacitor filled with a dielectric substance having a dielectric constant of 2. When compared to water, which has a dielectric constant of 80, the cell membrane is a much weaker dielectric material. Free charges therefore accumulate on both sides of the membrane, the normal resting potential difference across the membrane being 10 mV. The application of electric field pulses across the membrane however causes an increase in the trans-membrane potential, effecting attractive forces between the positive and negative charges on the opposite sides of the membrane to compress the membrane thereby reducing its thickness. Local breakdown of the membrane occurs when the applied electric field reaches a value sufficient to build a 1-V potential across the membrane. This breakdown is reversible when the size and number of the resulting pores are relatively small compared to the total membrane surface. Irreversible breakdown occurs at higher field strengths thus causing inactivation of the cell. A critical electric field strength ($E_c$) should be applied prior to the attainment of a trans-membrane potential of 1 V and cell

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inactivation. The value of $E_c$ varies with cell diameter (*13*). According to these authors, cells having a diameter of 1 μm require an $E_c$ value of 10 kV/cm to build 1 V transmembrane potential difference. Experimental data, however, demonstrate $E_c$ values in the range of 15 to 25 kV/cm for rod shaped cells with 2 μm in length and 1 μm in diameter.

## Relative Susceptibility of Microorganisms, Comparison with Conventional Technologies

The inactivation of microorganisms during processing by both conventional and novel technologies is influenced by treatment parameters, properties of the food and characteristics of the targeted microorganisms. The susceptibility of food microflora to a given set of processing parameters is dictated by the physical and compositional properties of the food, the genetic makeup of contaminating microorganisms and their physiological status.

The susceptibility of microorganisms to conventional processing has been extensively investigated. Some of the generalizations applicable to the relative susceptibility of microorganisms to these conventional technologies also apply to novel non-thermal processing methods. It is well established for example, that bacterial spores are resistant to all types of preservation processes, while vegetative bacterial cells are highly susceptible to most. Resistance to processing increases when microbial cells are at the stationary, rather than at the exponential phase of growth. In addition, microorganisms are generally more resistant to processing under conditions of low water activity, than under high water activity conditions. Food provides greater protection to microorganisms against inactivation by processing, than do simple microbiological media or buffers. Although these generalizations may illustrate the fundamental challenges that face the development of new food processing technologies, a more detailed picture and more comprehensive studies are required by the food industry in order to ensure the success of emerging non-thermal technologies.

Pulsed electric field technology is more suited to the processing of homogeneous liquid than particulate or solid foods. Conductivity (i.e., ability to conduct electric current), pH and water activity of food have profound effects on microbial inactivation by PEF. Food having a low conductivity, high water activity and an acidic pH is an ideal medium for effective PEF treatment.

Microorganisms show variation in their susceptibility to PEF on the basis of their size, cellular structure, and physiological status. Yeasts are generally more susceptible to PEF than are bacteria, while among the bacteria, rods appear to be more susceptible than cocci (*8*, *14*). On this basis, PEF therefore appears to be more effective against large microbial cells (Figure 1). This hypothesis was supported by a recent finding that PEF lacks efficacy against viral particles (*15*).

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Gram-positive bacteria have a peptidoglycan rich rigid cell wall while gram-negative bacteria have a less rigid but multilayered cell envelope. This difference in cellular structure may account for the greater susceptibility of Gram-negative bacteria to PEF. Susceptibility to PEF also increases when microbial cultures are at the exponential rather than at the stationary phase of growth (*4*).

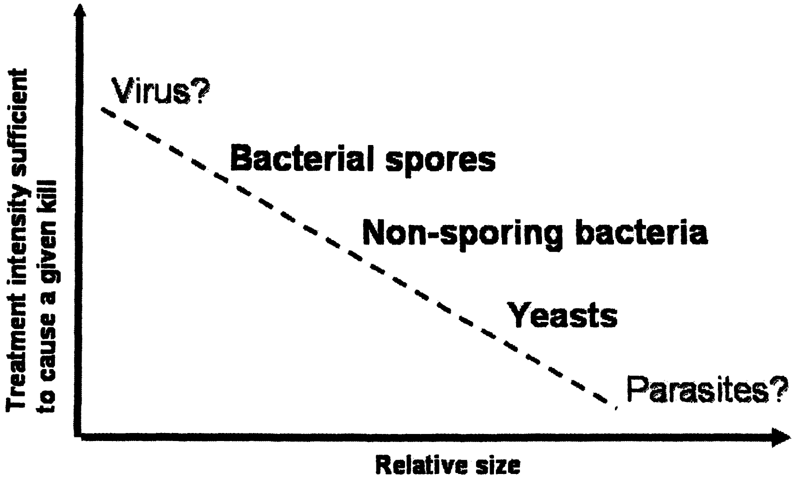

*Figure 1. Relative resistance of microorganisms to pulsed electric field (PEF) as a function of cell size. Current, data are not available about the susceptibility of viruses and parasites to PEF, but this model predicts resistance of the former and sensitivity of the latter (adapted from Yousef (16)).*

## Increasing the Efficacy of Pulsed Electric Field Technology through Combined Treatments

Heat is widely applied in food preservation because of its effectiveness against microorganisms, viruses and enzymes. The dose of heat applied in the preservation process can be adjusted to achieve almost any desired level of microbial safety provided the food quality remains acceptable. Nonthermal technologies, however, result in limited microbial lethality. At the highest doses currently feasible, nonthermal preservation methods cannot accomplish commercial sterility in most foods, owing to the resistance of bacterial spores. These technologies may however reduce the microbial risk associated with some foods to an acceptable level. Processes that eliminate 5 logs of *E. coli* O157:H7

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(e.g., pasteurization) may be considered adequate for the production of safe fruit juices (*17*). Pulsed electric field can be used to accomplish this goal (*18*).

Current nonthermal technologies may not be adequate to deliver treatments equivalent to pasteurization, in some low acid foods. Treating milk with PEF for 600 μs at 30 kV/cm and 25°C, for example, eliminated only 3 logs of *L. monocytogenes* (*19*). Pasteurization of milk commonly eliminates at least 5 logs of this pathogen (*20*). Limitations of nonthermal technologies can be overcome through the combination of treatments.

Relatively few studies have focused on the combined effect of PEF and other preservation methods. Liu et al (*21*) observed a synergistic effect between PEF and organic acids (sorbic and benzoic) against *E. coli* O157:H7 (Figure 2). Calderón-Miranda et al. (*22*) observed a greater inactivation rate when *L. innocua* was treated with PEF in the presence of nisin, compared with the PEF treatment alone (Figure 3). Inactivation of *L. monocytogenes* in milk was enhanced when PEF was applied at 50°C than at 25°C (*19*).

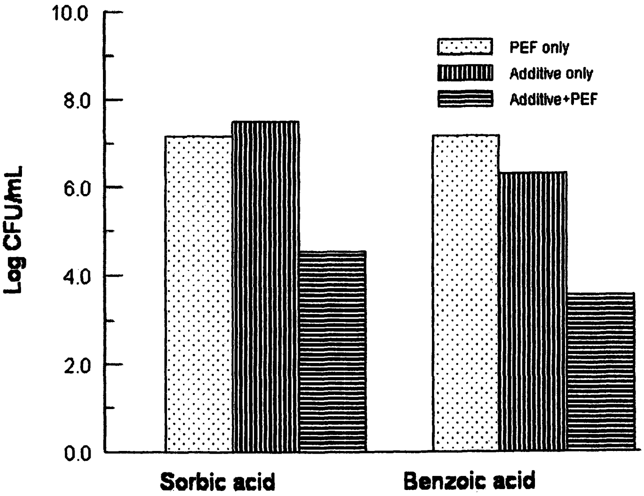

*Figure 2. Counts of Escherichia coli 0157:H7 after treatment with combinations of pulsed electric field (PEF) and food additives. Initial count was 2.5 × $10^8$ CFU/mL and additive concentration was 1000 ppm. (adapted from Liu (21).)*

Bacterial spores and fungal ascospores are resistant to pulsed electric fields. Grahl and Märkl (14) reported no inactivation, subsequent to treatment of endospores of *B. cereus* and *C. tyrobutiricum* and ascospores of *Byssochlamys nivea* with 22.4 kV/cm for up to 0.2 ms at temperatures of less than 45 to 50°C.

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Pagán et al. (*23*) used 75, 2-$\mu$s pulses at 60 kV/cm and 60°C against *B. subtilis* spores. No spore inactivation was observed, even when this treatment was combined with 5000 IU lysozyme/mL. Yin et al. (*24*) treated *B. subtilis* spores with 30 kV/cm for 1800 $\mu$s and obtained less than a one log decrease in spore viability. A similar treatment, but in the presence of a germinant (L-alanine), resulted in 2 log spore inactivations. Marquez et al. (*25*) suspended *B. cereus* spores in 0.1M NaCl solution and applied PEF at 25°C using an electric field of 50 kV/cm and 50 pulses. Contrary to all other findings reported, this treatment inactivated 5 logs of spores/mL.

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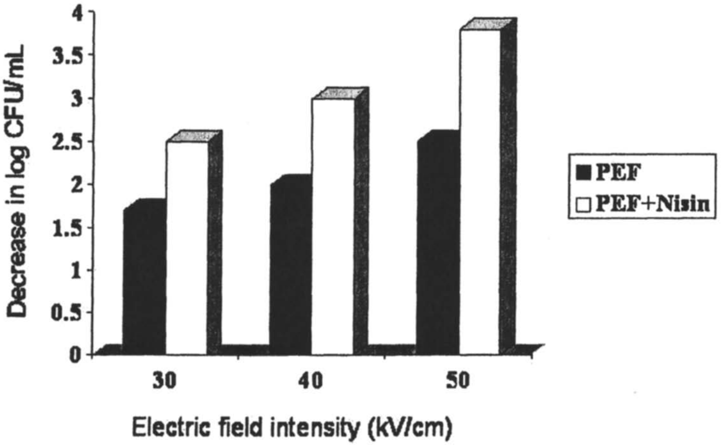

*Figure 3. Decrease in counts (log CFU/mL) of Listeria innocua in skim milk with or without 37.5 IU nisin/ml after treatment with pulsed electric field (PEF) at different electric field intensities (adapted from Calderón-Miranda (22)).*

## Measuring efficacy of pulsed electric filed treatment and use of surrogate microorganisms

The efficacy of a processing technology is ideally tested in food that has been inoculated with pathogens (challenge studies). The test pathogen to be used in these challenge studies varies in accordance with the food concerned. *Listeria*-inoculated milk and *Salmonella*-contaminated liquid egg for example are commonly used to test processes designed for the production of safely pasteurized milk and liquid egg, respectively (*26*, *27*). Pasteurized milk and liquid egg may be characterized as refrigerated low-acid foods. Shelf-stable, low acid foods (e.g., canned peas) are ideally tested using spores of *C. botulinum*. Until recently, high acid foods (e.g., fruit juices) were rarely tested with the use of pathogenic microorganisms since the main goal of processing is the elimination of aciduric spoilage microorganisms. Outbreaks of diseases due to the consumption of *E. coli* O157:H7-contaminated apple cider and juice (*28*, *29*) and *Salmonella*-contaminated orange juice (*30*) have however prompted the food industry to test pathogen-inoculated high-acid foods.

Challenge studies cannot be run in a commercial food processing facility since pathogen-contaminated foods require careful handling in specialized laboratories (e.g., Biosafety Level-II). However, in order to run these studies in such specialized laboratories, laboratory-scale or bench-top processing equipment that closely mimic the commercial processing line is needed. Scarcity of such equipment is another hurdle to be overcome in order to conduct challenge studies. The food industry has therefore been searching for "surrogate" microorganisms to allow safe testing of new technologies under real processing conditions. Surrogate microorganisms or "surrogates" are non-pathogenic microorganisms which show similarity with the targeted pathogen in its susceptibility to the processing technology. Ideal surrogates are (a) easy to culture in the laboratory, (b) easy to isolate on selective media and to enumerate on both selective and non-selective media, and (c) stable in morphological and biochemical properties. *C. sporogenes* PA 3679 has been effectively used as a surrogate to *C. botulinum* in heat inactivation studies. *L. innocua* has been used to study treatments that target *L. monocytogenes* (*31*). Fratamico et al. (*32*) constructed non-pathogenic strains of *E. coli* O157:H7 for use in challenge studies. The new strains carry the luciferase (*luc*) and the green fluorescent protein (*gfp*) genes. The recombinant *E. coli* strains were similar to their parent strains in biochemical and immunological assays and growth kinetics, yet easily detectable using fluorescence techniques. Industry may still be reluctant to use such surrogates in food processing facilities. The presence of these surrogates in the processing environment may result in false positives when environmental samples from these facilities are tested for the presence of pathogens.

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## Ensuring the Safety of Food Processed by Pulsed Electric Field

The application of nonthermal technologies in food processing should be preceded by extensive studies in order to ensure the safety of the treated food. These studies should determine both the critical treatment parameters and the magnitudes of these parameters that are sufficient to eliminate predefined levels of targeted pathogens. As discussed earlier, the critical processing parameters in pulsed electric field technology include electric field intensity (kV/cm), treatment time ($\mu$s), and product temperature (°C).

The targeted pathogen depends on the food in question. Raw milk for example, occasionally causes listeriosis in humans. This disease is caused by *L. monocytogenes*, which is naturally present in raw milk at levels that normally do not exceed $10^2$ CFU/mL. A non-thermal process designed to produce safe milk (i.e., cold pasteurization) should be applied at an intensity that is adequate to eliminate at least 5 logs of *L. monocytogenes* (*20*). Such a treatment ensures that the processing of raw contaminated milk results in a product containing less than one *Listeria* organism per kg. It is important to caution that the previous example over simplifies a complicated safety question. The process just described minimizes appreciably, but does not totally eliminate the risk of listeriosis in processed milk. In addition to *L. monocytogenes*, other pathogens (e.g., *Mycobacterium* spp.) are of concern in raw milk. If *L. monocytogenes* is more resistant to the non-thermal process than other pathogens, then treatments designed to minimize the risk of listeriosis should be adequate to significantly reduce other microbial risks. Studies should therefore be conducted to determine the relative susceptibility of pathogens of concern in a given food, to a non-thermal process. The most resistant pathogen should be considered as the target of the process.

Having established the critical process parameters and the targeted pathogen, a validation method should follow. Validation entails inoculation of the food with the targeted pathogen and treatment under conditions similar to an actual processing run. If the non-thermal processing equipment is located in a commercial processing facility, pathogens may be substituted with suitable surrogate microorganisms. The non-thermal treatment is applied to inoculated food at pre-calculated levels of critical process parameters. Validation is accomplished if the non-thermal processing treatment decreases the population of the targeted pathogen (or its surrogate) below the pre-defined level.

Validation of a non-thermal process substantiates the feasibility of using the technology in commercial applications. An increasingly popular approach to ensure the safe commercial production of food is the hazard analysis critical control point (HACCP) system (*33*). The use of HACCP in non-thermal food processing is recommended. Essential steps in developing a HACCP plan with emphasis on the non-thermal process include:

1. Assessing potential hazards (microbial, chemical or physical) associated with the non-thermally processed food (see earlier discussion).

2. Determining critical control points (CCPs) required for control of the recognized hazards. The non-thermal processing step (e.g., application of electric pulses) along the production line are typical CCPs.

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3. Establishing critical limits at each CCP. An upper and lower limit should be defined for each critical process parameter, and food must be kept within these limits during non-thermal processing.

4. Developing and setting up procedures to monitor the CCP. In PEF processing, the pulse application step is a CCP that can be monitored by an oscilloscope (to measure field intensity, pulse width and pulse rate), a flowmeter (to measure product flow rate) and a temperature sensor (to measure product temperature before and after the treatment).

5. Corrective actions should follow if the critical limits are breached during food processing. Where process deviations occur, the flow of any under-processed product can be diverted by a valve for reprocessing.

6. Establishing procedures to verify the control of hazards. The absence of the targeted pathogen in non-thermally processed food verifies the safe processing.

7. Establishing effective record keeping. Values of critical process parameters for each production run must be recorded and retained for future reference.

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## Economic Considerations

Currently no commercial food processing operations are reliant on PEF technology. The fruit juice market is likely to be the first to benefit from this technology. The PEF technology is inherently suitable for continuous operation and large product flow rate. Although high initial costs constitute a significant obstacle in applying PEF in commercial operations, operating costs are low (*4*).

## Conclusion

Interest in nonthermal food processing technologies has increased appreciably in the past decade. These technologies promise to maintain the critical balance between safety and marketability of a new generation of foods. Pulsed electric field technology can be used to produce safe fresh-like acid foods (e.g., fruit juices), but extensive research is needed to adapt this technology for the production of shelf-stable low acid foods. Current limitations of PEF technology may be overcome when combined with conventional preservation methods.

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## Chapter 12

# Antibiotic Activities of Plant Compounds against Non-Resistant and Antibiotic-Resistant Foodborne Human Pathogens

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As part of an effort designed to develop antimicrobial food formulations that will protect both the food and the consumer against pathogenic bacteria and viruses, we evaluated the bactericidal activities of ~300 plant essential oils, oil constituents, phenolic benzaldehydes, and benzoic acids, and polyphenolic catechins against the foodborne pathogens *Bacillus cereus, Campylobacter jejuni, Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enterica,* and *Staphylococcus aureus.* This brief overview summarizes some of our results. The data show that many of these phytochemicals are highly active against both non-resistant and antibiotic-resistant bacteria a pH 7 buffer and in a pH 3.7 apple juice. To help define the mechanism of antibacterial effects of phytochemicals, we also describe exploratory studies on the spontaneous fluorescence (autofluorescence) and ATP release of *E. coli,* to assess the sub-lethal effects of carvacrol. Future studies should define their antimicrobial effectiveness in human foods and animal feed and as disinfectants of surfaces of fruits and vegetables and of non-food items such as cutting boards.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

## Introduction

Food processors, food safety researchers, regulatory agencies, and the general public have been increasingly concerned with the growing number of foodborne illness outbreaks caused by some pathogens. The increasing antibiotic resistance of some pathogens associated with foodborne illness is another concern. Therefore, there has been increasing interest in developing new types of effective and non-toxic antimicrobial compounds.

Numerous foodborne diseases are syndromes that result from ingesting foods that are contaminated with either infectious microorganisms or toxic substances (toxins) produced by microorganisms. Foodborne pathogenic bacteria include *Bacillus cereus, Campylobacter jejuni, Clostridium botulinum, Clostridium perfringens, Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica.* Bacteria can exert adverse effects in tissues of animals and humans in at least two ways: adhesion to cells and release of cellular toxins. Understanding the molecular basis of the action of bacteria and of bacterial toxins in vitro and in vivo will facilitate devising appropriate food-compatible strategies to inactivate pathogenic bacteria and their toxins.

### Research Objectives

The primary objective of our research effort is to develop hitherto unexplored ways to reduce the human pathogen burden of foods with the aid of naturally occurring, plant-derived antimicrobial compounds. Specifically, the research (a) explores antimicrobial properties of pure compounds and of plant extracts; (b) determines the composition of extracts; (c) delineates chemical structure-antimicrobial activity relationships; (d) assesses additivity/synergism of mixtures of active phytochemicals; (e) evaluates bactericidal activities against antibiotic-resistant bacteria; and (f) evaluates effectiveness in human foods and animal feeds. The ultimate goal of these studies is to develop a better understanding of structural features of plant-derived compounds that govern antibacterial activities as well as devising food formulations that use the active compounds to reduce pathogens in foods, feeds, and possibly also in animals and humans after consumption. Studies with foods will assess the safety, solubility, and sensory properties including color and flavor of the test compounds as well as effects of the food matrix and of storage temperatures and times on antibacterial activities.

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## Bactericidal Activities ($BA_{50}$ values)

Bactericidal activities are defined as the percentage of a test compound that kills 50% of the bacteria, determined as follows. Each compound was tested at a series of six dilutions from in the mixture. The colony-forming units (CFU values) from the concentration-antibiotic activity experiments were transferred to a Microsoft Excel 8.0 Spreadsheet. The number of CFU from each dilution was matched with the average control value to determine the % of bacteria killed per well. Each of the dose-response profiles (% test compound versus % bactericidal activity) was examined graphically and the $BA_{50}$ values were estimated by a linear regression. The lower the $BA_{50}$ or the higher the $1/BA_{50}$ value, the higher the activity. The bactericidal assay was simple to perform and the results were reproducible in assays with the same samples tested at different times.

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## Antibacterial Plant Essential Oils and Oil Compounds

Plant essential oils are a potentially useful source of antimicrobial compounds. The general objective of our studies were (a) to screen 120 naturally occurring and food-compatible plant-derived oils and oil compounds for their antimicrobial activities against four species of bacterial foodborne pathogens; and (b) to identify compounds structural features in the oil constituents (Table I) that may be responsible for the bactericidal activities (*1*).

The ten most active oils were:

- *Campylobacter jejuni* ($BA_{50}$, 0.003-0.009): Marigold, ginger root, jasmine, patchouli, gardenia, cedarwood, carrot seed, celery seed, mugwort, spikenard, and orange bitter.
- *Escherichia coli* O157:H7 ($BA_{50}$, 0.046-0.14): Oregano, thyme, cinnamon, palmarosa, bay leaf, clove bud, lemon grass, and allspice.
- *Listeria monocytogenes* ($BA_{50}$, 0.057-0.092): Gardenia, cedarwood, bay leaf, clove bud, oregano, cinnamon, allspice, thyme, and patchouli.
- *Salmonella enterica* ($BA_{50}$, 0.045-0.14): Thyme, oregano, cinnamon, clove bud, allspice, bay leaf, palmarosa, and marjoram.

The ten most active oil compounds (constituents) were:

- *Campylobacter jejuni* ($BA_{50}$, 0.003-0.034): Cinnamaldehyde, estragole, carvacrol, benzaldehyde, citral, thymol, eugenol, perillaldehyde, carvone R, and geranyl acetate.

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**Table I. $BA_{50}$ Values of Oils/Oil Compounds Active in Four Pathogens**

| *Oil/oil compound* | *E. coli* | *S. enterica* | *C. jejuni* | *L. monocytogenes* |
|---|---|---|---|---|
| Cinnamaldehyde | 0.06 | 0.04 | 0.003 | 0.01 |
| Thymol | 0.06 | 0.03 | 0.02 | 0.08 |
| Oregano Spanish | 0.05 | 0.05 | 0.01 | 0.08 |
| Carvacrol | 0.06 | 0.05 | 0.01 | 0.09 |
| Oregano Origanum | 0.05 | 0.05 | 0.02 | 0.10 |
| Eugenol | 0.11 | 0.09 | 0.02 | 0.08 |
| Cinnamon Leaf | 0.13 | 0.08 | 0.03 | 0.09 |
| Thyme | 0.05 | 0.05 | 0.02 | 0.22 |
| Bay Leaf | 0.13 | 0.13 | 0.03 | 0.07 |
| Clove Bud | 0.13 | 0.13 | 0.02 | 0.09 |
| Allspice | 0.14 | 0.13 | 0.02 | 0.08 |
| Cinnamon Bark | 0.18 | 0.14 | 0.02 | 0.08 |
| Cinnamon Cassia | 0.11 | 0.07 | 0.01 | 0.15 |
| Lemon Grass | 0.14 | 0.16 | 0.02 | 0.12 |
| Palmarosa | 0.12 | 0.14 | 0.07 | 0.27 |
| Citral | 0.22 | 0.23 | 0.02 | 0.20 |
| Basil | 0.41 | 0.42 | 0.02 | 0.12 |
| Perillaldehyde | 0.27 | 0.20 | 0.03 | 0.30 |
| Salicylaldehyde | 0.13 | 0.12 | 0.04 | 0.45 |
| Geraniol | 0.15 | 0.15 | 0.10 | 0.51 |
| Estragole | 0.28 | 0.21 | 0.01 | 0.35 |
| Fir Needle | 0.48 | 0.61 | 0.01 | 0.08 |
| Elemi | 0.40 | 0.44 | 0.01 | 0.22 |
| Orange Mandarin | 0.41 | 0.64 | 0.01 | 0.10 |
| Cumin Seed | 0.30 | 0.36 | 0.10 | 0.25 |
| Carvone S | 0.48 | 0.39 | 0.04 | 0.17 |
| Spearmint | 0.28 | 0.29 | 0.03 | 0.57 |
| Caraway | 0.46 | 0.47 | 0.03 | 0.24 |
| Citronella | 0.41 | 0.49 | 0.09 | 0.18 |
| Hyssop | 0.57 | 0.41 | 0.10 | 0.18 |
| Nutmeg | 0.55 | 0.44 | 0.18 | 0.20 |
| Benzaldehyde | 0.48 | 0.36 | 0.02 | 0.36 |
| Lavender | 0.41 | 0.41 | 0.06 | 0.34 |
| Rose French | 0.43 | 0.50 | 0.05 | 0.29 |
| Rose Geranium | 0.41 | 0.40 | 0.09 | 0.32 |
| Rose Damask | 0.55 | 0.44 | 0.11 | 0.36 |
| Wormwood | 0.55 | 0.52 | 0.38 | 0.10 |
| Coriander | 0.40 | 0.48 | 0.08 | 0.50 |
| Menthol | 0.53 | 0.50 | 0.40 | 0.48 |

- *Escherichia coli* H7O157 ($BA_{50}$, 0.057-0.28): Carvacrol, cinnamaldehyde, thymol, eugenol, salicylaldehyde, geraniol, isoeugenol, citral, perillaldehyde, and estragole.
- *Listeria monocytogenes* ($BA_{50}$, 0.019-0.43): Cinnamaldehyde, eugenol, thymol, carvacrol, citral, geraniol, perillaldehyde, carvone S, estragole, and salicylaldehyde.
- *Salmonella enterica* ($BA_{50}$, 0.034-0.21): Thymol, cinnamaldehyde, carvacrol, eugenol, salicylaldehyde, geraniol, isoeugenol, terpineol, perillaldehyde, and estragole.

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A number of associations were observed from comparisons of the chemical structures of the purified plant compounds and their antimicrobial activities. Both the aldehyde compounds (cinnamaldehyde, citral, citronellal, perillaldehyde, and salicylaldehyde) and phenolic compounds (carvacrol, eugenol, and thymol) were very active. The antibacterial activities of isomeric compounds eugenol/isoeugenol were significantly different. Eugenol was about 13 times more active than isoeugenol against *C. jejuni* and *Listeria*.

Analysis of the oils by HPLC showed that the bactericidal results are related to the composition of the oils (Table II) (*2*). These studies provide information about new ways to protect apple juice and possibly other foods against contamination by human pathogens.

The flavor and taste of oils can vary widely (spicy cinnamon oil, mild oregano oil). Specific oils would be more compatible than others for food categories such as fruits and vegetables, juices, milk, cheese, poultry, and red meat. The antimicrobial activities under food processing conditions such as baking, cooking, frying, and microwaving are mostly unknown. The most active compounds provide candidates for future studies of flavor, taste, and antibacterial activity in foods.

## Antibacterial Activities of Phenolic Benzaldehydes and Benzoic Acids

We evaluated the bactericidal activities of 35 benzaldehydes, 35 benzoic acids with 0, 1, 2, or 3 hydroxy (OH) and/or methoxy ($OCH_3$) groups attached to different position on the benzene rings in a pH 7.0 buffer against *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica*. Table III shows some of our results (*3*). Of the 70 compounds tested, 24 were found to be active against all four pathogens. *C. jejuni* was ~100 times more sensitive than the other three pathogens. Comparison of chemical structures of the test compounds and their activities revealed the following: (a) activity of the aldehyde (CHO) > carboxyl (COOH) group without or with the presence of OH groups; (b) activity of compounds with trisubstituted-OH > disubstituted-OH

> monosubstituted-OH; (c) activity of compounds with OH > $OCH_3$; (d) compounds with mixed OH and $OCH_3$ groups exhibited variable results, i.e. in some cases $OCH_3$ groups enhanced activity and in others they did not. Figure 1 shows bactericidal activities ($1/BA_{50}$ values) of the top ten compounds against each of the following four pathogens: *C. jejuni*, *E. coli*, *L. monocytogenes*, and *S. enterica*.

The fact that many of the active phenolic compounds evaluated are soluble in an aqueous solution should facilitate their use in a variety of food systems.

**Table II. Components in plant essential oils measured by HPLC**

| *Oil* | *Component* | *% in oil ± SD (n=3)* |
|---|---|---|
| Clove | Eugenol | 86.5 ± 0.38 |
| Cinnamon | Cinnamaldehyde | 85.7 ± 0.95 |
| Lemongrass | Citral | 86.1 ± 1.98 |
| Palmarosa | Geraniol | 85.0 ± 1.70 |
| Bitter orange | Perillaldehyde | 0.06 ± 0.003 |
| Bitter orange | Limonene | 87.1 ± 1.07 |
| Mandarin orange | Perillaldehyde | 0.10 ± 0.009 |
| Mandarin orange | Limonene | 33.8 ± 1.12 |
| Sweet orange | Limonene | 90.0 ± 1.00 |
| Tangerine oil | Limonene | 82.9 ± 3.45 |
| Lime oil | Limonene | 38.5 ± 2.86 |
| Grapefruit oil | Limonene | 82.6 ± 4.45 |
| Lemon | Limonene | 61.6 ± 1.59 |

## Antibiotic Activities of Catechins

A bactericidal assay was used to evaluate dose-responses of a dilution series in a pH 7.0 buffer of carvacrol and thymol and 11 green tea phenolic compounds (catechins) (*4*). Carvacrol and thymol had similar $BA_{50}$ values of 0.022 and 0.025, respectively. Six of the eleven catechins evaluated in a bactericidal assay were active against the foodborne pathogen *Bacillus cereus* in the assay, with average 30 min $BA_{50}$ values ranging from 0.031 for (–)-gallocatechin to 0.0000048% for (–)-epicatechin gallate, a 6,500 variation from least to most active catechin. Activities of the catechins were in the following order: (–)-epicatechin gallate > epigallocatechin-3-gallate = (–)-gallocatechin gallate > (–)-catechin gallate >> (–)-gallocatechin = (–)-epigallocatechin. Future studies should define their antimicrobial effectiveness in foods and as disinfectants of surfaces of fruits and vegetables, baked products, poultry, and meat products, and of non-food items such as cutting boards.

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**Table III. Bactericidal activities of phenolic benzaldehydes and benzoic acid compounds active against all four pathogens (*Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica*)**

| | *Average $BA_{50}$ %* |
|---|---|
| 2,4,6-Trihydroxybenzaldehyde | 0.026 |
| 2,5-Dihydroxybenzaldehyde | 0.042 |
| 2,3-Dihydroxybenzaldehyde | 0.067 |
| 2,3,4-Trihydroxybenzaldehyde | 0.069 |
| 2-Hydroxy-5-methoxybenzaldehyde | 0.090 |
| 2-Hydroxy-3-methoxybenzaldehyde | 0.136 |
| 4-Hydroxy-2,6-dimethoxybenzaldehyde | 0.144 |
| 2,5-Dihydroxybenzaldehyde | 0.146 |
| 2, 4-Dihydroxybenzaldehyde | 0.163 |
| 2-Hydroxybenzaldehyde | 0.166 |
| 2-Hydroxy-4-methoxybenzaldehyde | 0.174 |
| 2-Hydroxy-5-methoxybenzaldehyde | 0.205 |
| 3,4,5-Trihydroxybenzoate methyl ester | 0.220 |
| 3-Hydroxybenzaldehyde | 0.272 |
| 3,4-Dihydroxy-5-methoxybenzaldehyde | 0.276 |
| 4-Hydroxybenzaldehyde | 0.330 |
| 4-Methoxybenzaldehyde | 0.330 |
| Benzaldehyde | 0.336 |
| 2-Methoxybenzaldehyde | 0.362 |
| 2,3-Dimethoxybenzaldehyde | 0.430 |
| 2,6-Dimethoxybenzaldehyde | 0.442 |
| 5-Hydroxy-3,4-dimethoxybenzaldehyde | 0.452 |
| 4-Hydroxy-3-methoxybenzaldehyde | 0.463 |
| 2,3,4-Trimethoxybenzaldehyde | 0.585 |

## Antibiotic Activities Against Antibiotic-Resistant Bacteria

Antibiotics are widely used as animal feed supplements to fight infections, to promote growth of livestock and poultry, and to reduce production costs. Antibiotics are also used in horticulture as aerosols to fruit trees for controlling bacterial infection. Resistant microorganisms often arise from administration of sub-therapeutic levels of antibiotics in animal feeds. There is a need to develop new inexpensive alternatives for standard antibiotics that can be effective against antibiotic-resistant bacteria. We evaluated selected natural products (Figure 2) for their ability to inhibit growth of three antibiotic-resistant organisms (*5*). Table IV shows some of the results with oregano oil.

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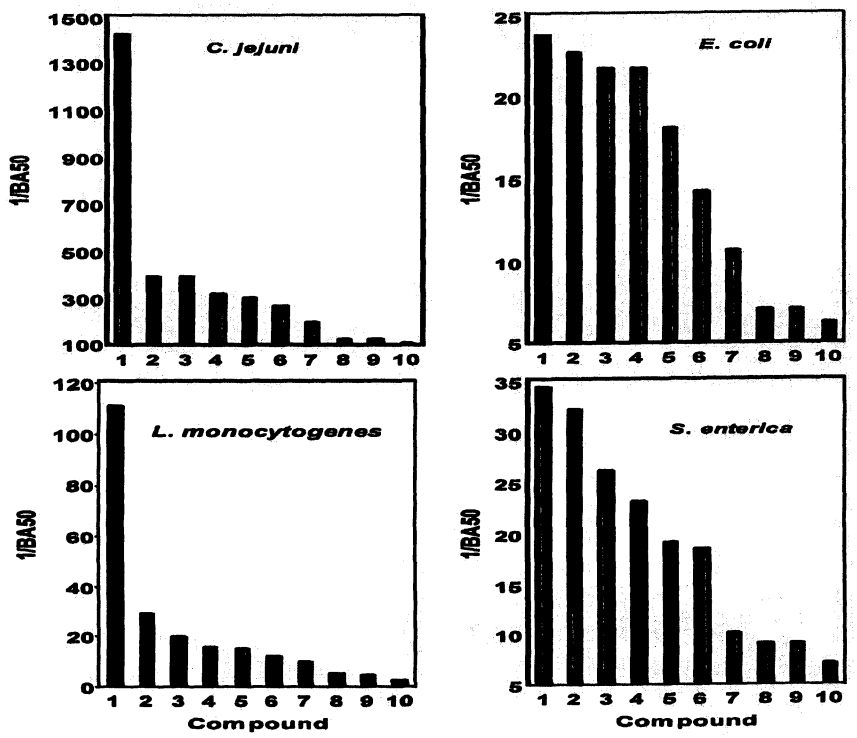

*Figure 1. Comparison of bactericidal activities (1/$BA_{50}$ values) of the top ten phenolic compounds against each of the following four pathogens:* ***Campylobacter jejuni****: 1. 2-hydroxy-5-methoxybenzaldehyde (highest activity); 2. 3,4,5-trihydroxybenzaldehyde; 3. 2-hydroxy-4-mthoxybenzaldehyde; 4. 3,4-dihydroxybenzaldehyde; 5. 3,4-dihydroxy-5-methoxybenzaldehyde; 6. 2,3,4-trihydroxybenzaldehyde; 7. 3,4,5-trihydroxybenzoic acid; 8. 4-hydroxy-2,6-dimethoxybenzaldehyde; 9. 2,4,6-trihydroxybenzaldehyde; 10. 3,4,5-trihydroxybenzoate methyl ester.* ***Escherichia coli O157:H7****: 1. 3,4,5-trihydroxybenzaldehyde; 2. 2,3,4-trihydroxybenzaldehyde; 3. 4-hydroxy-2,6-dimethoxybenzaldehyde; 4. 2,5-dihydroxybenzaldehyde; 5. 2,4,6-trihydroxybenzaldehyde; 6. 2-hydroxy-5-methoxybenzaldehyde; 7. 2,3-dihydroxybenzaldehyde; 8. 2,4-dihydroxybenzaldehyde; 9. 2-hydroxybenzaldehyde; 10. 3,5-dihydroxybenzaldehyde.* ***Listeria monocytogenes****: 1. 2,4,6-trihydroxybenzaldehyde; 2. 2-hydroxy-4-methoxybenzaldehyde; 3. 2,3-dihydroxybenzaldehyde; 4. 2,5-dihydroxybenzaldehyde; 5. 3,5-dihydroxybenzaldehyde; 6. 2-hydroxy-3-methoxybenzaldehyde; 7. 2-hydroxy-5-methoxybenzaldehyde; 8. 3,4,5-trihydroxybenzoate methyl ester; 9. 2,3,4-trihydroxybenzaldehyde; 10. 2,4-dihydroxybenzaldehyde.* ***Salmonella enterica****: 1. 2,3,4-trihydroxybenzaldehyde; 2. 2,4,6-trihydroxybenzaldehyde; 3. 3,4,5-trihydroxybenzoic acid; 4. 2,5-dihydroxybenzaldehyde; 5. 4-hydroxy-2,6-dimethoxybenzaldehyde; 6. 3,4,5-trihydroxybenzaldehyde; 7. 2,4-dihydroxybenzaldehyde; 8. 2,3,4-trihydroxybenzoic acid; 9. 2,3-dihydroxybenzaldehyde; 10. 3-methoxybenzaldehyde.*

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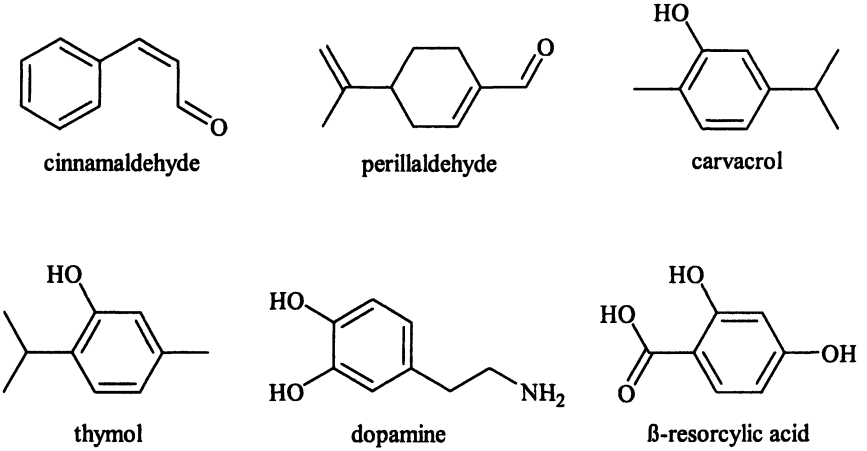

*Figure 2. Structures of compounds active against antibiotic-resistant bacteria.*

After demonstrating the lack of effectiveness of standard antibiotics against acquired antibiotic resistance of ***Bacillus cereus*** (NCTC10989), ***Escherichia coli*** (NCTC1186), and ***Staphylococcus aureus*** (ATCC12715), we showed that the following natural substances were antibacterial against these resistant pathogens: cinnamon oil, oregano oil, thyme oil, carvacrol, (S)-perillaldehyde, 3,4-dihydroxybenzoic acid (β-resorcylic acid), and 3,4-dihydroxyphenethylamine (dopamine). Exposure of the three pathogens to a dilution series of the test compounds revealed that oregano oil was the most active substance. The oils and pure compounds exhibited exceptional activity against *B. cereus* vegetative cells, with oregano oil being active at nanograms/ml levels. In contrast, activities against *B. cereus* spores were very low. Activities of the test compounds were in the following approximate order: oregano oil > thyme oil » carvacrol > cinnamon oil > perillaldehyde > dopamine > β-resorcylic acid. The order of susceptibilities of the pathogens to inactivation was: *B. cereus* (vegetative) » *S. aureus* » *E. coli* » *B. cereus* (spores). Some of the test substances may be effective against antibiotic-resistant bacteria in foods and feeds.

The results show that all test substances were active and some were highly active against the antibiotic-resistant organisms. The major objective of these studies is to develop candidates for incorporation into formulations that use these compounds to reduce both non-resistant as well as antibiotic-resistant pathogens in human foods and animal feeds.

**Table IV. Antibiotic Activities of Oregano Oil Against Antibiotic-Resistant Bacteria**

| *Oregano oil µg/ml* | *Bacillus cereus % kill* | *Escherichia coli % kill* | *Staphylococcus aureus % kill* |
|---|---|---|---|
| 66.7 | 100 | 100 | 100 |
| 6.67 | 100 | 94.8 | 94.4 |
| 1.34 | 99.7 | 32.1 | 0 |
| 0.667 | 100 | 11.5 | 0 |
| 0.335 | 96.5 | | |
| 0.170 | 81.9 | | |
| 0.0083 | 67.7 | | |
| 0.0042 | 36.2 | | |

In a related study, we found that the bactericidal activities of the above-mentioned test substances against an antibiotic resistant *Micrococcus luteus* strain (*6*). Unlike many standard antibiotics, all were active against this organism. Since the compounds evaluated in the present study were active against both 'non-pathogenic' antibiotic-resistant *M. luteus* bacteria as well as against pathogenic strains of *B. cereus*, *E. coli*, and *S. aureus*, future studies of the effectiveness of new antibiotic agents with the resistant *M. luteus* strain may predict their effectiveness against resistant pathogenic bacteria.

The availability of new, plant-derived antibiotics provides more options for treatment of livestock and poultry and reduces the exposure of humans to resistant bacteria.

## Antibacterial Activities of Plant Essential Oils and their Components in Apple Juice

The use of heat and irradiation with fruit juices can both kill bacteria that are present and induce compositional and other changes in the juice. These include increase in the mutagen count as measured by the *Salmonella typhimurium* (Ames) test (*7, 8*), formation of undesirable Maillard browning products (*9*), as well as damage to vitamin C and other adverse effects (*10*). Irradiation is reported to induce the formation of malanodialdehyde, which is reported to induce hemolysis of human red blood cells (*11*). These effects and the widespread production and consumption of unpasteurized apple juices, especially those designated organic juices, suggest the need to develop additional effective, food-compatible, safe formulations to protect both the juice and therefore the consumer against infection by human pathogens. Non-toxic,

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food-compatible natural products are one source of compounds that may provide a useful intervention for combating food pathogens.

To help overcome this problem, we evaluated 17 plant essential oils and 9 oil compounds for antibacterial activity against the *E. coli* O157:H7 and *S. enterica* in apple juices in a bactericidal assay in terms of % of the sample that resulted in a 50% decrease in the number of bacteria ($BA_{50}$) (*2*). Figure 3 depicts selected results.

The ten compounds most active against *E. coli* (60 min $BA_{50}$ range in clear juice, 0.018 to 0.093) were: carvacrol, oregano oil, geraniol, eugenol, cinnamon leaf oil, citral, clove bud oil, lemongrass oil, cinnamon bark oil, and lemon oil. Against *S. enterica* ($BA_{50}$ range 0.0044 to 0.011) the compounds were: Melissa oil, carvacrol, oregano oil, terpineol, geraniol, lemon oil, citral, lemongrass oil, cinnamon leaf oil, and linalool.

The following are novel and unexpected aspects of our observations: (a) a number of food-compatible oils such as lemon were much more active in apple juice than in pH 7 buffer; (b) some apple varieties possibly with a high phenol content appear to contain antimicrobial compounds; (c) structure-antimicrobial activity studies permit selection of the most active individual compounds as well mixtures of antimicrobials exhibiting synergism for application to foods; and (d) the antimicrobial formulations we developed may also be active against human pathogens reported to infect other liquid foods such as orange and tomato juices and milk as well as against spoilage organisms, including bacteria and fungi.

Our findings complement earlier cited efforts to protect apple juice against contamination by human pathogens as well as reported antimicrobial effectiveness of essential oils in other foods including carrots, salads, and meat, poultry and fish. They also will facilitate commercial use of highly active compounds to protect apple juice against infection.

The results suggest some areas for further studies with some of the food-compatible compounds. Some possibilities include adding active oils or their active components during a stage in the production and storage of apple juice and/or adding a few drops of one of the oils to unpasteurized juice before consumption and monitoring the effects on the microbial flora including pathogens.

## Mechanistic Aspects - Effects of Carvacrol on Autofluorescence and ATP Release of *E. coli*

Ultee et al. (*12*) showed that the consequences of exposing the foodborne pathogen *B. cereus* to carvacrol include depletion of the intracellular ATP pool, change in membrane potential, and increase in permeability of the cytoplasm membrane for protons and potassium ions The loss of the ion gradient is

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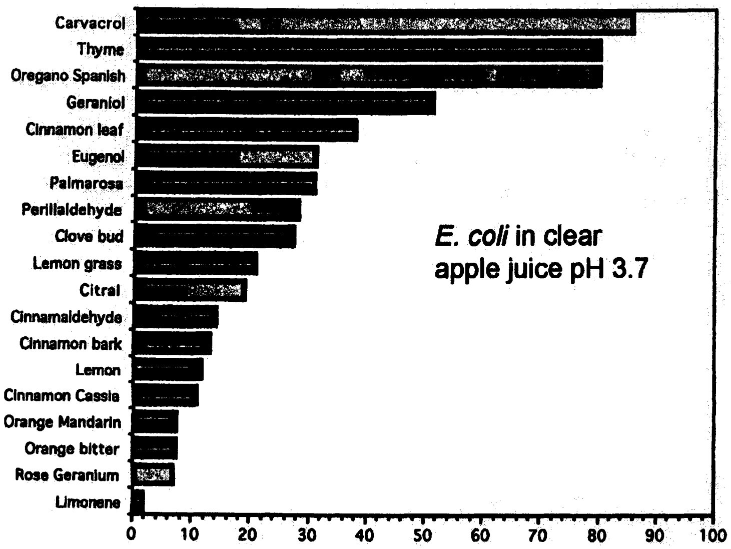

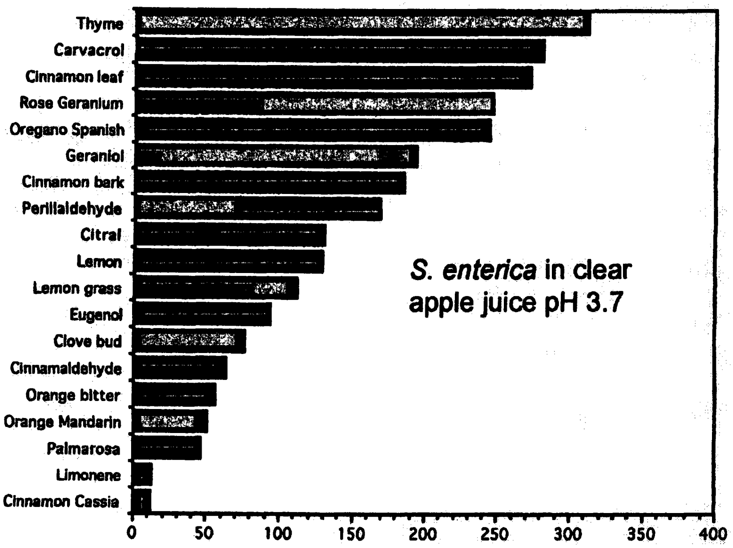

***Figure 3.** Relative bactericidal activities (sum of 1/BA$_{50}$ values determined at 5 min and 60 min) of active oils and oil compounds against E. coli and S. enterica in clear apple juice.*

responsible for loss of essential metabolic processes in the cell and consequently cell death. This and related studies utilized standard methods of screening in which gross numbers of organisms are used to determine the most effective dose. As part of an effort to help define the mechanisms of antimicrobial action of botanicals, we utilized autofluorescence spectra to determine the effect of carvacrol on *E. coli* (*13*).

An autofluorescence spectrum is the result of the sum of fluorescence spectra contributed by each fluorescing component of a microorganism. We utilized three-dimensional autofluorescence, a composite of hundreds of two-dimensional spectra, to determine the effect of carvacrol on *E. coli*. Because the composition of each microorganism is unique, each microorganism will generate a unique autofluorescence spectrum (Figure 4, 5).

The autofluorescence data showed significant changes at much lower concentrations of carvacrol (0.01 mM) than changes in membrane potential or release of ATP (ATP flux) after disruption of the bacterial cell membrane (1-2 mM) (Figure 6), suggesting that the autofluorescence detects physiological responses to carvacrol more efficiently than do changes in membrane potential or of release of ATP. This study shows that the technique we developed to measure autofluorescence in *E. coli* differentiates between autofluorescence associated with native bacterial cells and those exposed to an antimicrobial agent such as carvacrol. Examination of the difference spectra (Figure 5) shows that the fluorescent patterns (components that autofluoresce) of the bacterial cells are affected by the levels of carvacrol. The results suggest that autofluorescence has the potential not only to identify specific microorganisms but possibly also to assess their viability and cellular activities. If dead bacteria exhibit different autofluorescence patterns than non-viable and dead ones, it may be possible to automate the autofluorescence technique to measure bacteristatic and/or bactericidal activities of numerous natural and synthetic antimicrobial compounds. This aspect merits further study.

The following procedures, described here for the first time, were used to determine the autofluorescence spectra. A monochromator-based spectrofluorimeter (Photon Technology Inc, Princeton, NJ) was used for fluorescence detection. This instrument uses a xenon arc lamp to illuminate a one-half meter monochromator. The output of the monochromator is focused on a sample chamber wherein a sample cuvette is placed. Emission from the sample cuvette was collected at an angle of 90 degrees to the excitation after passing through an emission monochromator. Collection of the data was performed using photon-counting and a Hammamatsu R920 photomultiplier tube. Photon counts were stored on magnetic media and later analyzed and plotted using S-Plus (Insightful, Seattle, WA) and Prism. A digital filter was applied to the raw data to remove photon scatter less than 25 nM, the absolute value of the excitation wavelength less the emission wavelength. An additional digital filter was applied

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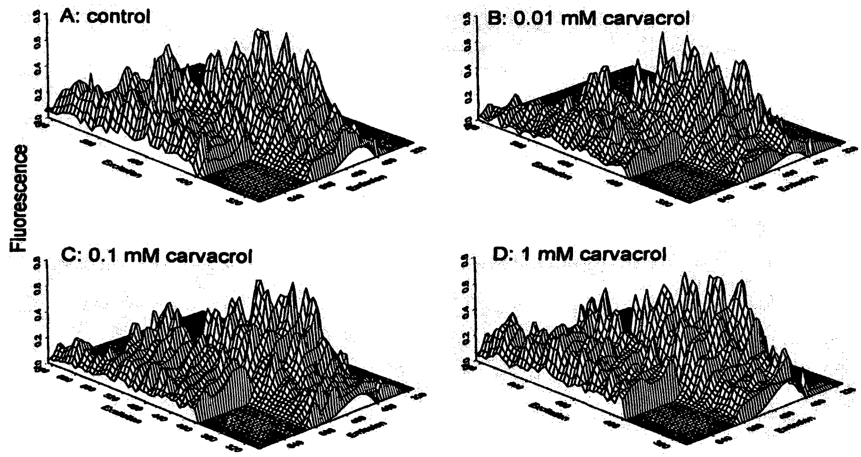

*Figure 4. Three-dimensional representation of autofluorescence of* E. coli *C600.*

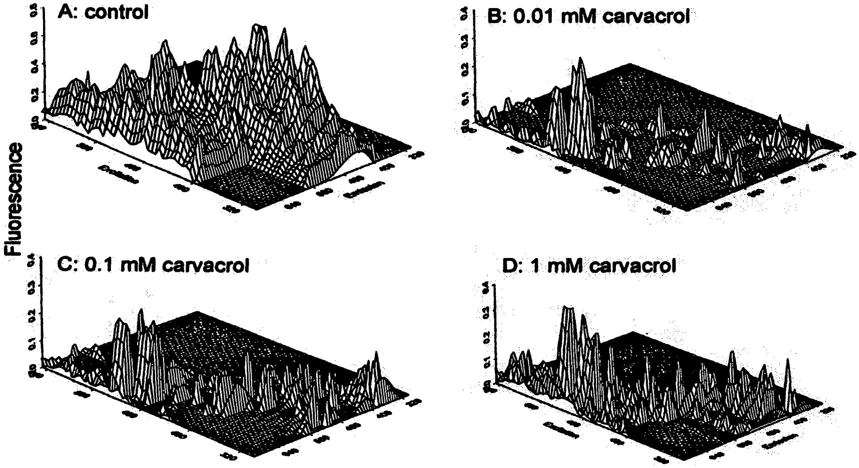

*Figure 5. Three-dimensional autofluorescence of E. coli – difference spectra between untreated and carvacrol treated bacteria*

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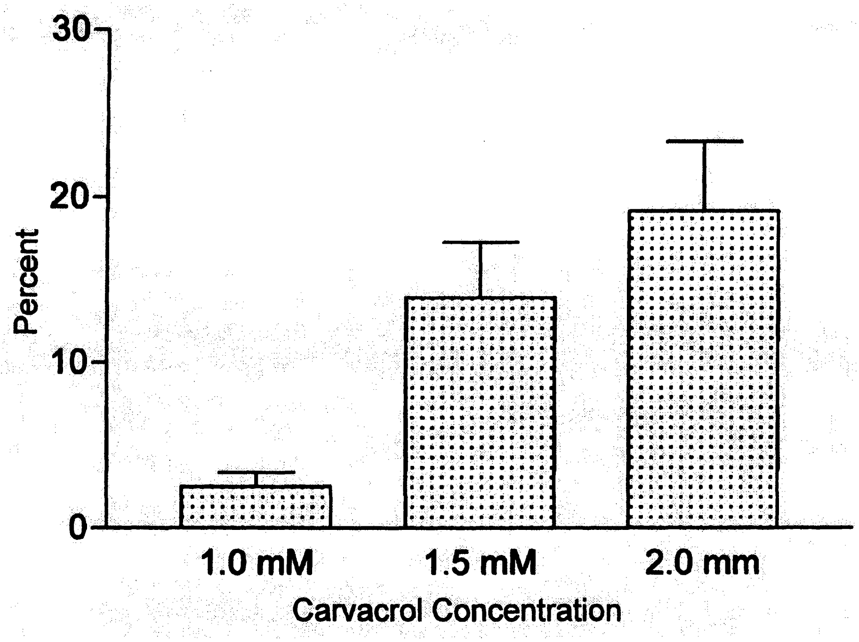

*Figure 6. Effect of concentration of carvacrol on percent ATP release from E. coli C600*

to the data to remove the emissions from doubling of the primary excitation wavelength. After 15 min of incubation at room temperature in either control or carvacrol treatment, the cuvette containing the bacterial sample was placed in the sample chamber of a spectrofluorimeter and fluorescence was measured using excitation wavelengths of 300-700 nm and 400-700 nm emission. Fluorescence data was acquired by a computer, stored on magnetic media, and processed as three-dimensional plots using S-Plus (Insightful, Seattle). All fluorescence scans were referenced to a factory fluorescent calibration standard.

The following procedure was used to determine carvacrol-induced ATP release from *E. coli*. Cells were diluted to an optical density $A_{660}$ of 1.0 with fresh Trypticase Soy Broth (TSB) containing 15% glycerol. The diluted culture (1 mL) was centrifuged at 2k × g for 5 min in a 1.5 mL centrifuge tube. This spin was repeated for each individual carvacrol treatment. The pellet was then resuspended in 200 µL of 50 mM HEPES, 10mM $MgSO_4$, 1 mM EDTA at pH 7.8. The bacterial suspension was then transferred to a 1 mL cuvette and the

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cuvette was inserted in the photon counter sample chamber. The reagents were then added to the cuvette in the following order: 200 µL 25 mM Acetyl CoA in 50 mM HEPES, 10 mM $MgSO_4$, 1 mM EDTA at pH 7.8. The photon counter was started immediately after 400 µL luciferin-luciferase was injected into the cuvette. Photons were counted for 300 sec before 200 µL of carvacrol, 50 mM HEPES, 10mM $MgSO_4$, 1 mM EDTA, 10 % EtOH at pH 7.8 was added to the cuvette. Bacteria were tested with three treatments of carvacrol: 1.0, 1.5, or 2.0 mM. Photon counts were then collected for 15 min before the addition of 200 µL of ATP releasing agent with phosphatase inhibitor (Turner Designs, Sunnyvale, CA) was added and photon emission was collected for an additional 15 min. Data was collected for a total of 2300 sec at 500 millisecond intervals. Results are reported as the percent of ATP released during treatment from total ATP in the cell as determined by the releasing agent. Differences between treatments are represented as the mean ± standard error of the mean.

Studies are needed to define the following additional aspects of antimicrobial activities of phytochemicals and plant extracts: (a) antibacterial and antiviral activities of natural anthocyanins, capsaicins, flavonoids, theaflavins, and glucosinolates; (b) additive and synergistic effects of mixtures of highly active compounds; (c) activities against antibiotic-resistant *Campylobacter jejuni*, *Clostridium perfringence*, *Escherichia coli* O157:H7, *Salmonella enterica*, *Listeria monocytogenes*, and foodborne pathogenic viruses; and (d) applications to foods, feeds, and as disinfectants (*14-17*).

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I thank Carol E. Levin for assistance with the preparation of this manuscript and my colleagues whose names appear on the cited references for excellent scientific collaborations.

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## Chapter 13

# Uses and Limits of Microbial Testing

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Microbiological testing is an integral part of most microbiological food safety problems, making up a significant amount of the effort of many food microbiologists. Effective use of these analytical tools requires both a thorough understanding of the technical details of methods being employed and how the performance of the methods is influenced by sampling limitations. However, too often the latter is incompletely evaluated or understood. In particular, improvements in the sensitivity and specificity of new methods can be effectively lost if it is accompanied by a decrease in sample size. The basis for sampling requirements, particularly when a microorganism of concern is present at low levels, is based on the probability of detecting the specific portion of food being tested from a larger population of food portions. Two general approaches based on statistical requirements are most often employed in the testing of food samples, "within-batch testing" and "between-batch testing." The purpose of with-in batch testing is to establish that a specific lot of food is "safe" in regard to a specific hazard at a specified level of confidence. This approach assumes no prior knowledge of the history of the food. Practical sampling considerations generally limits its effectiveness to batches where more than 1% of the samples are contaminated. Often referred to as process control testing, the purpose of between-batch testing is

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ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

verification that a process is operating as intended. This type of testing assumes that there is extensive knowledge of the system being evaluated. This approach can be very sensitive to changes above an established baseline, however, that sensitivity is again dependent on the number of samples taken and the inherent number of contaminated portions produced by the system. An understanding of which tool to employ and the limitations of those tools are critical to the effective use of microbiological testing.

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An essential component of virtually every microbial food safety program worldwide is the periodic testing of foods to assess microbiological quality and/or safety. These programs are initiated, in part, based on the intuitive feeling of consumers, industry and food control agencies alike that microbiological testing provides data critical to determining if the food supply is safe. In fact, microbial testing can be an extremely useful and powerful tool when used effectively and appropriately (*1*,*2*). However, the effective use and interpretation of microbial testing schemes is dependent on both the providers and the users of the results having a clear understanding of the scientific and statistical principles underlying such testing, including the basic assumptions that are inherent every time a food sample is analyzed. Regretfully, the basic training for individuals that conduct or use the results of microbial food testing too often does not provide the type of indepth consideration of the characteristics of microbial testing schemes that is needed to design testing schemes that provide the required data in a cost effective manner. The purpose of the current manuscript is to provide a brief overview of key attributes, principles, underlying assumptions and decisions that need to be considered in establishing microbial testing programs. Of necessity, this review will focus on introducing key concepts and parameters, and will not get into the details of the various decision tools that are available. These are available through a variety of references. In particular, the International Commission on Microbiological Specifications of Foods (ICMSF) has been instrumental in articulating the principles for microbiological testing (*3*).

The critical phase in any microbial food safety testing program is the initial design phase. It is during that phase that decisions must be reached and documented in relation to the testing program's goal(s), underlying basic assumptions, required stringency, criteria for interpreting results in relation to subsequent actions that should be taken, and unique characteristics of the food and microorganisms that will affect interpretation of the results. Some of the key questions that need to be asked during the design phase include:

- What are the microorganisms of concern that affect the quality or safety of the food?
- What are the sources of contamination, conditions, or activities that lead to the microorganism(s) of concern being present at unacceptable levels or frequencies?
- What conditions (both uses and abuses) are the foods likely to experience once they have left the manufacturer's control?
- What is the purpose of each proposed assay?
- What are the methods that will be employed and what are their performance characteristics (e.g., lower limit of detection, repeatability, ruggedness, variance)?
- What information is provided by each proposed assay?
- What are the acceptance/rejection criteria for each proposed assay and what are the actions that will be taken as a result of these results?
- What are the consequences of mistakenly releasing foods that should have been rejected?
- What are the consequences of mistakenly rejecting foods that were actually within required specifications?
- What are the basis and limits associated with any proposed use of a surrogate microorganism in relation to the pathogen and/or condition that it is being used to assess?

Articulation and documentation of these and related questions are an important tool in developing a microbiological testing program. It helps ensure that the testing program meets the needs, provides a blueprint to those implementing the program, and serves as a historical record for future evaluation of program effectiveness. These questions help ensure that the critical thinking and decision-making needed for an effective testing program is achieved. The current chapter will focus on key decisions related to the design of testing programs and sampling plans. A detailed consideration of specific methods applications (e.g., PCR, ELISA, culture techniques) will not be covered, but standard references are available for the interested reader.

## Why Test?

As stated above, one of the key questions in designing a microbiological testing program is the reason for performing the microbiological assays. Too

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often the reason for testing is not clearly defined, which can lead to the incorrect design of testing programs or the incorrect interpretation of results obtained from them. The reasons for most microbiological testing fall into one of five areas:

- Characterization of a food and/or a food control system to establish a "microbial history,"
- Determination of the microbiological safety or quality of a specific lot/batch,
- Verification that a food control system is operating as intended,
- Environmental testing, and
- Investigational testing.

Each of these testing types has a different purpose and different assumptions and statistical basis. For example, lot testing is based on the assumption that the tester has no advanced knowledge of the history of the lot being examined, and also on the assumption that the presence of microorganisms in the product may or may not have specified distribution (e.g. log normal). Conversely, process control testing to verify that a food control system is operating as intended presumes that the tester has extensive knowledge of how the product was produced, and is largely determining if there has been an alteration in one of the basic parameters or assumptions.

Briefly, the purposes of the types of testing programs can be summarized as follows:

- Characterization of a food control system. These are testing programs undertaken to establish the performance characteristics of a food product and the manufacturing system used for its production. This type of testing is typically done prior to the initiation of a new product or method of manufacture and establishes the baseline data what can be expected when the production system is "under control." The characterization process may require the collection of data over an extended period, particularly if there are seasonal differences associated raw ingredients or product manufacture, distribution, marketing, or use.
- Testing of lots/batches for safety. This is the traditional testing of single lots of food against a specified criterion, and is often the basis for decisions related to whether a consignment of food is or is not safe. As noted above, this assumes that the tester has no knowledge about the history of the product. The effectiveness of this approach is largely limited by the large number of samples required to provide the high degree of assurance typically required when the level of "contaminated units" within a lot is small.

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- Verification that a food control system is operating as intended. Often referred to as "process control testing," this approach is based on "between-lot" testing to evaluate whether a system is operating as originally designed. The focus of this type of testing is on the process and not on verifying that any single lot of product has achieved a microbial safety or quality. This approach typically requires extensive knowledge of the microbiological history of the production system.
- Environmental testing. This type of testing is employed most often to determine if a food production facility is continuing to follow good hygienic practices. It is most effective when used in conjunction with a well established baseline microbiological history against which individual results can be compared. When used in relation to food safety decisions, there is an underlying assumption that there is a relationship between the incidence of pathogens (or a surrogate microorganism) in the environment and in the final product.
- Investigational testing. This is a broad category of microbiological testing approaches that may be employed when a problem (i.e., loss of control) occurs. The purpose of such testing is to identify the source and cause of the problem so that it can be corrected. Investigational testing can range from the simple (e.g., swaps in processing area) to the highly sophisticated (e.g., molecular subtyping of the isolates from environmental swabs). Investigational sampling is also an integral part of epidemiological investigations when there has been an adverse event involving foods. Typically, there are fewer statistical guidelines with investigational testing.

Since one of the primary goals of the current chapter is to provide an overview of the issues that have to be addressed during the design and implementation of testing programs and sampling plans, only within-lot testing and process control (between-lot) testing will be discussed further. More information on the other types of microbiological testing programs can be obtained from standard references (*3*).

In considering the two types of testing that will be discussed further, a term that will be used is "lot." There appears to be no formal definition of lot, nor are there specific guidelines for the establishing the size of lots within production runs. ICMSF (*3*) recommended that "Ideally, a lot is a quantity of food or food units produced and handled under uniform conditions." Often lots are established in terms of production runs on specific lines during a limited or specified period of time. Regulatory agencies often define lots in relation to potential recalls as the period of time between complete cleanups of a manufacturing facility. Increasingly, food manufacturers are being required to more rigorously define and maintain lot identity in order to facilitate potential recalls.

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## Where to Test?

Traditionally, the manufacture of a food product is envisioned as a series of sequential steps that begins with raw ingredients and ends with a finished product that is ready to be introduced into the marketplace. In recent years, our improved understanding of the epidemiology of foodborne disease, the globalization of the food industry and changes in the marketing and consumption of foods have increasingly required that the food chain be extended conceptually and practically to encompass all of the steps from the production of agricultural commodities on the farm to the consumption of the final product in the home.

The ICMSF (*3*) conceptually viewed the performance of an entire food safety control system as being described by the equation:

$$H_0 - \Sigma R + \Sigma I \leq FSO$$

Where $H_0$ is the initial level of contamination, $\Sigma R$ is the sum of all the steps that reduces that level of microbial contamination, $\Sigma I$ is the sum of all the steps that increase that level of microbial contamination, and FSO (food safety objective) is the overall stringency that the food safety control system is expected to achieve in order to meet public health goals. The Codex Alimentarius Committee of Food Hygiene has expanded on this approach concepts by introducing the concept of Performance Objective, the maximum level or frequency of a hazard that can occur at a specific step in the food chain and still meet the overall FSO for the entire food safety system. Thus, the PO articulates the required level of stringency at a specific step in a food chain, and can be conceptually expressed by the equation:

$$H_0 - \Sigma R + \Sigma I \leq PO$$

These concepts are graphically presented in Figure 1. Once established, the achievement of a PO can be verified through the establishment of performance criteria, process criteria, product criteria, or microbiological criteria.

The important point that these concepts have in relation to microbiological testing programs is that the results of microbiological assay at a specified point in the food chain is an integrated measure of all of the steps preceding it. Sampling of raw ingredients at the loading dock measures $H_0$ for the manufacturer but also is the integrated measure of the level of control for all steps prior to receipt such as adherence to good agricultural practices, effective harvesting or slaughter operations, and control of distribution conditions. Sampling at an intermediate point in the processing of a product again integrates the entire performance of the food safety control system up to that point. If one was trying to measure the performance of the entire food safety control system

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from "farm to fork," one would have to perform microbiological testing on food just prior to it being eaten by the consumer. While totally impractical, it would capture all of the steps in production, processing, distribution, marketing and preparation chain.

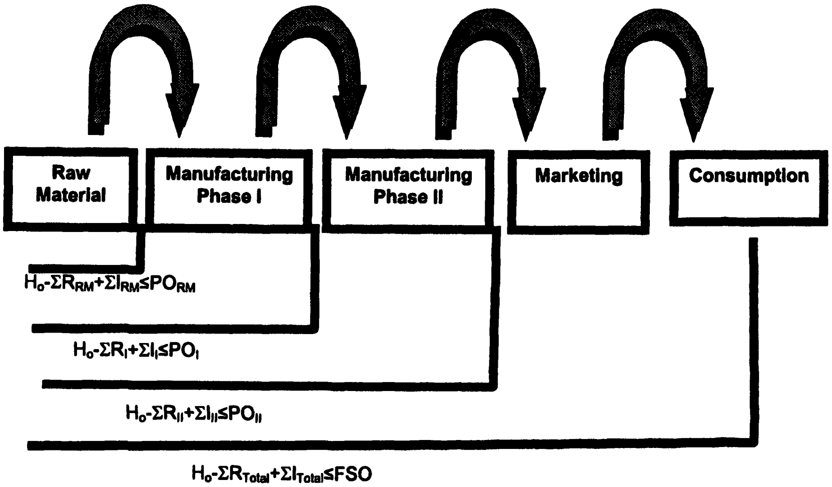
Raw Material
Manufacturing Phase I
Manufacturing Phase II
Marketing
Consumption
$H_o-\Sigma R_{RM}+\Sigma I_{RM}\leq PO_{RM}$
$H_o-\Sigma R_I+\Sigma I_I\leq PO_I$
$H_o-\Sigma R_{II}+\Sigma I_{II}\leq PO_{II}$
$H_o-\Sigma R_{Total}+\Sigma I_{Total}\leq FSO$

*Figure 1. Graphical representation that the location at which a microbiological sample is taken reflects the integrated impact of all the increases and decreases in microbial contamination that affected the food and its ingredients prior to step in the food chain where the sample was taken.*

The sampling of product just prior to it entering commerce is commonly referred to as end-product testing. Industry, consumers and food control agencies often give microbiological testing at this point special significance because this location is often associated with the activities of food control agencies. However, it is conceptually no different than sampling at any other locations, i.e., it measures the integrated effects of all the steps that preceded this point. Thus, if one is restricted to examining a single location in the entire food manufacturing chain, end-product sampling has its benefits. However, if the primary determinant for microbiological safety or quality is further up or down the food chain, then alternative sampling sites would likely be more informative. While it requires more sampling, the most effective testing programs examine the food chain in several locations. The benefit of multiple sampling locations is that if key sites are selected this greatly facilitates the rapid identification and

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correction of a problem when it is detected. For example, if four sites in a food chain are monitored, raw ingredients, intermediate step A, intermediate step B, and final product, and only the final product and step B give aberrant results, then the subsequent evaluation can rapidly focus on the activities that take place between step A and step B.

The specific sites for microbiological testing will vary with the pathogen, the food, and the method of manufacture. In general, sites should be selected based on the usefulness of the information that will be obtained and the likelihood that there will be a meaningful result upon which an action will be taken. For example, microbiological testing after a highly reliable heat treatment that effectively eliminates microorganisms of concern will typically be a waste of time and resources since the likelihood of detecting a fail with microbiological testing that would not more easily be detected with physical measurements (e.g., recording thermometer) is exceedingly small. Conversely, steps where contamination or a loss of control are reasonably likely may warrant consideration as potential sampling locations in a microbiological testing program.

## How Often to Test?

The simple answer to the question, at the risk of sounding trite, is "as often as necessary but no more than that." The frequency of testing is again dependent on the reason underlying the testing program. If the purpose of the testing program is to differentiate acceptable vs. unacceptable lots of food in regard to safety, then the frequency of testing will be every lot of food. Conversely, if the purpose of the testing program is to verify the effectiveness of a food safety control system, then there will likely be a scheduled sampling scheme wherein samples are taken at a pre-determined rate. In all cases, it is important to consult an appropriately trained statistician to determine the "level of confidence" achieved with the sampling frequency selected and compare that with the consequences of not detecting a loss of control. For example, taking one or two sample per month is not likely to provide any realistic assurance of process control. Conversely, taking an excess number of samples beyond that needed to meet a pre-determined "level of confidence" is likely to be expensive but offer little increased confidence.

## Within-Lot Testing

As indicated above, the traditional use of microbiological testing programs, particularly for foods being presented at a "port of entry," is lot testing, where

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the frequency or level of a pathogen or indicatory microorganisms was determined and compared against a pre-established microbiological criterion, be it a standard, a guideline, or a specification. The purpose of this type of testing is to determine if a specific lot of food is acceptable. This is achieved by using an established sampling plan wherein a specified number of sample units are removed from the lot and examined using a standard protocol. The specific portion of each sample unit that is actually examined is commonly referred to as the analytical unit and is usually expressed in terms of a specified mass or volume (e.g., 25 g, 10 ml). Proper drawing of the samples is important to avoid bias in the sampling scheme. Unless there is advance knowledge to the contrary, random selection of sample units from the lot is an important means of avoiding sampling bias and obtaining a representative sample. However, if the contamination within a lot is non-randomly distributed than a representative sample may not be best obtained by random selection. There are alternative sampling schemes, such as stratified random sampling approaches that can be used to combine random sampling within non-random examination of sub-lots. Stratification is a means for handling known sources of variation such as the non-random contamination of a lot.

An important concept in understanding the statistical basis for microbiological testing is the concept of "percentage of defectives." This is the portion of samples of a specified mass or volume that contained a frequency or level of microorganism of concern that was deemed unacceptable. For example, suppose a lot of food consisted of 10,000 g and the entire lot of food was divided into 1 g portions and examined for *Salmonella*. If 10 of the10,000 1-g samples were positive than for that lot, the percent defectives is 10/10,000 = 0.1%. Alternatively, if the number of positive samples had been 1000, then the % defectives would have been 10%. However, an entire lot of food would virtually never be examined since nothing would be left to consume. In almost all instances only a small subset of the potential sample units would actually be examined. However, this requires that the uncertainty associated with random sampling be taken into account in relation to the likelihood that part of the lot contains unacceptable levels of the microorganism of concern. For example, suppose we employed a sampling plan that examined ten 1-g samples of the lot described above. If the defect rate was 10%, there is a reasonably likelihood that one would detect that some of the lot exceeded the pre-established microbiological criterion. On the other hand, if the defect rate was 0.1%, there is little likelihood that this sampling plan would detect the fact that the lot contained sample units that exceeded the pre-established microbiological criterion. The specific probabilities of correctly accepting (or rejecting) a lot of food based on an established sampling plan can be calculated as a function of the percentage of defectives in a lot. This relationship serves as the basis for generating "operating characteristics curves" (OC curve) for a sampling plan. An example of an OC curve for a simple attribute sampling plan is depicted in Figure 2.

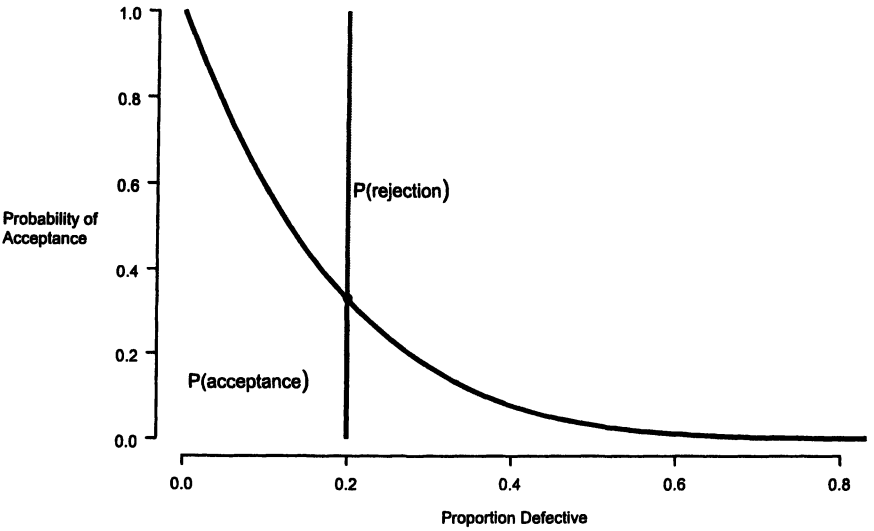

*Figure 2. Example of an "Operating Characteristics" (OC) curve depicting the probability of acceptance of a lot as a function of percent defective sample units for a 2-class attribute sampling plan with n = 5 and c = 0 (Adapted from ICMSF, 2002).*

Within-lot microbiological testing approaches are divided into broad types, "variables" testing and "attribute" testing, with the latter being further subdivided into two subcategories, 2-class and 3-class attribute plans. Variables testing is used in conjunction with quantitative data and is based on determining if the mean concentration and/or variance of a lot differs from a pre-established microbiological criterion. This approach to within-lot testing is not used extensively in conjunction with foods, and will not be discussed further. An introduction to this type of sampling plan can be found by consulting ICMSF (*3*) or other standard references.

Attribute testing is the primary approach used to assess the within-lot microbiological safety or quality. This is used in conjunction with "presence/absence" data or "binned" (stratified) quantitative data (e.g., < 10 CFU/g vs. ≥ 10 CFU/g). The simplest attribute plans are 2-class plans where the analytical units are examined for the presence of a specified microorganism or its occurrence above a specified concentration (which by convention is denoted by

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*m*) (see Figure 3) and compared against pre-established decision criteria. With 2-class sampling plans, two parameters must be articulated: $n$ = the number of analytical units of a specified size to be examined, and $c$ = the number of analytical units that can be "positive" and the lot still be considered acceptable (e.g., no more than 2 analytical units out of 10 exceed total aerobic plate counts of 1000 CFU/g).

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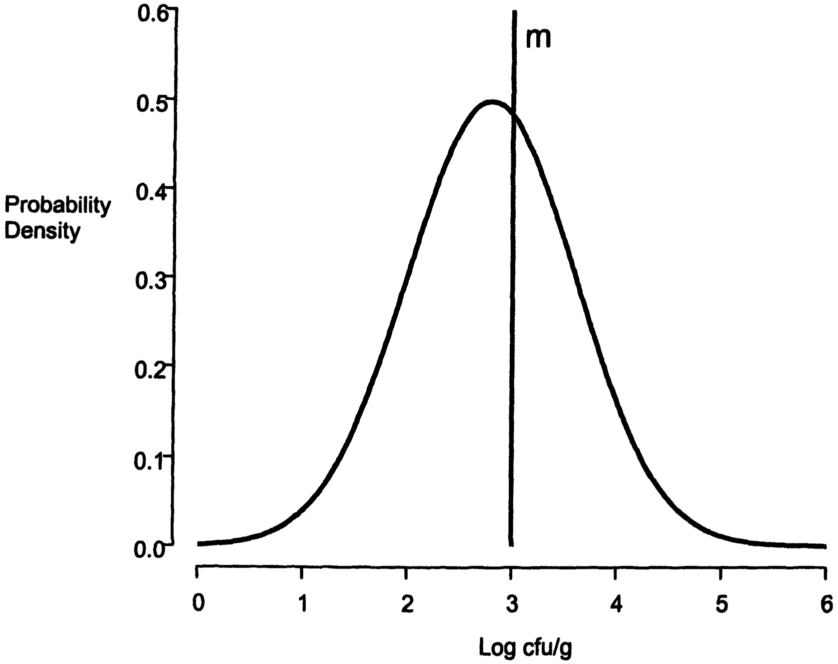

*Figure 3. Graphical representation of a 2-class attribute decision criterion (m) versus the hypothetical distribution of a microorganism of concern within a lot. In this example, samples units having Log (CFU/g) $\geq$ 3.0 would be considered unacceptable based either on binned quantitative or qualitative data where the method had a lower limit of detection of 1000 CFU/g. (Adapted from ICMSF, 2002)*

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The discriminatory power of a within-lot microbiological testing program is dependent on a number of factors related to analytical methodologies, particularly the methods lower limits of sensitivity. However, in most instances where standard methods are employed and the level of contamination is reasonably low, the primary factor affecting detection is the design sampling plan. The three parameters that determine the ability of a sampling plan to detect a contaminant is the number of sample units examined (*n*), the number of defective samples permitted among the sample units tested (*c*), and the size of the analytical unit being examined. Examples of how *n* affects the likelihood of detecting unacceptable lots and their ability to correctly distinguish between acceptable and unacceptable lots are depicted in Figure 4. Particularly in the case of presence/absence tests, the size of the analytical unit directly affects the lower limit of sensitivity of a method and thus the *m* value for the sampling plan. Thus, if the mean concentration of a pathogen in a food is 1 per 10 g, the likelihood of detecting it with a presence/absence assay in a single sample if the analytical unit is 1 g is approximately 10%. However, if the sample size was increased to 25 g, the probability of detecting the pathogen would approach 100%. In this instance, manipulating the size of the analytical unit effectively changes the lower limit of detection for the analysis and thus the *m* value for the sampling plan.

As introduced above, the percent of defective samples is an important determinant of the discriminatory power of a sampling plan. The percent of defective samples also posed significant practical limits for microbiological testing schemes. The relationship between n and percent defective on the discriminatory power of a sampling plan to correctly identify lots containing unacceptable portions is depicted in Table I. It is apparent that a practical limit for microbiological testing is faced when applied to lots with defective rates of ≤ 2%. Microbiological testing in those instances is generally impractical due to the large number of samples required to achieve a reasonable level of confidence. In some instances it may be possible to pool large numbers of sample units such as is done for the testing of eggs for *Salmonella* Enteritidis where the percent of defective eggs is in the range 1 per 10,000, however, even there the level of confidence is limited. On the other end of the scale, presence/absence testing of lots where the normal percent of defectives is high (e.g., ≥ 30%) is also of limited discriminatory value. It would be impractical to produce foods where even with a single sample, more than a third of the lots would be expected to be rejected. In those instance the manufacturer would need to consider alternative means for producing the product, or in those instances where the level of a hazard is important, switching to a quantitative sampling scheme and establishing decision criteria based on binned data (e.g., acceptable: ≤ 100 CFU/g).

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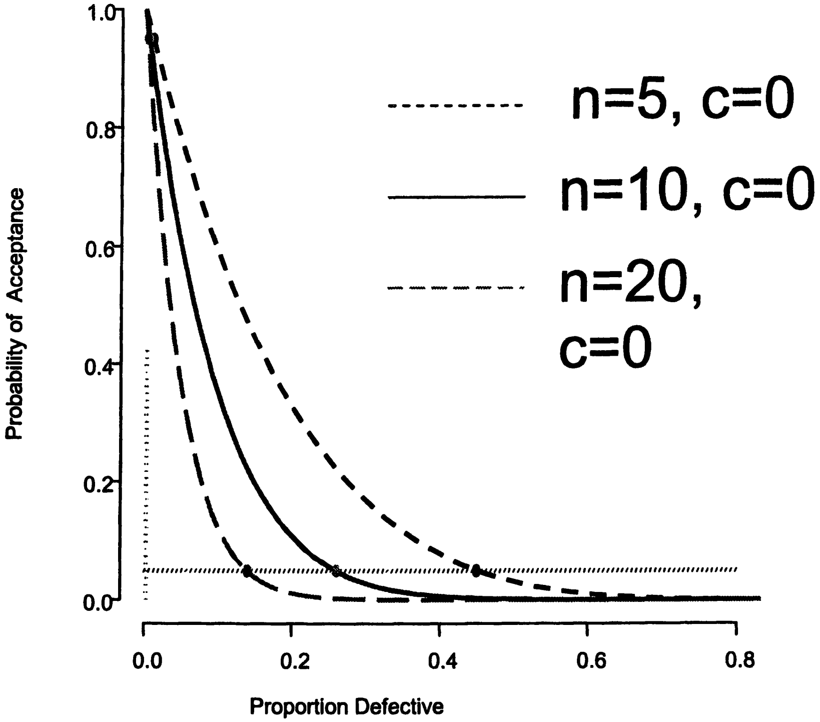

*Figure 4. Examples of the effect of number of sample units (n) examined in a 2-class sampling attribute sampling plan on the probability of accepting a lot as a function of the percent defective units within the lot. (Adapted from ICMSF, 2002)*

While presence/absence data are qualitative in nature, they can be used to estimate the levels of a microorganism within a lot if the distribution of the microorganism can be deduced. The distribution of microorganisms within a lot of food has generally been found to be log normally distributed, i.e., the level of a microorganism expressed as a log number is normally distributed (*3*). When this distribution can be assumed and there is historical data on the standard deviation of the distribution, the % defective rate can be used to estimate the mean log concentration within a lot. Conceptually, this is similar to the approach used to assign most likely concentration values when performing a Most Probable Number analysis except that one is performing it with a single dilution and a larger number of "tubes." This concept is useful since it allows confidence intervals for different sampling plans to be calculated based on the mean log concentration of the microbiological agent within the lot and provides a relative measure for evaluating the relative effectiveness of different sampling plans. For example, selected "ICMSF cases" (*3*) for a 2-class attribute sampling plan as a function of mean log concentration are depicted in Table II.

**Table I. Probability of Accepting a Lot as a Function of the Percentage of Defective Sample Units and the Number of Sample Units Examined. (Adapted from ICMSF (2002)).**

| *%* | *Number of sample units (n) tested from a lot* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Defective* | *3* | *5* | *10* | *15* | *20* | *30* | *60* | *100* |
| 2 | 0.94 | 0.90 | 0.82 | 0.74 | 0.67 | 0.55 | 0.30 | 0.13 |
| 5 | 0.86 | 0.77 | 0.60 | 0.46 | 0.36 | 0.21 | 0.05 | 0.01 |
| 10 | 0.73 | 0.59 | 0.35 | 0.21 | 0.12 | 0.04 | <0.005 | <0.005 |
| 20 | 0.51 | 0.33 | 0.11 | 0.04 | 0.01 | <0.005 | <0.005 | <0.005 |
| 30 | 0.34 | 0.17 | 0.03 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| 40 | 0.22 | 0.08 | <0.01 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| 50 | 0.13 | 0.03 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| 60 | 0.06 | 0.01 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| 70 | 0.03 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| 80 | 0.01 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| 90 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |

**Table II. Examples of Sampling Plan Performance for Selected "ICMSF Cases" as a Function of Calculated Mean Concentrations (or Greater) Such that a Lot Would Be Rejected with 95% Confidence with a 2-class Sampling Plan of m = 25 g and c = 0.[a] (Adapted from ICMSF, 2002)**

| *Case Number* | *Number of 25 g Sample Units Examined (n)* | *Mean Microbial Concentration* |
|---|---|---|
| 10 | 5 | 1 CFU/32 g |
| 11 | 10 | 1 CFU/83 g |
| 12 | 20 | 1 CFU/185 g |
| 13 | 15 | 1 CFU/135 g |
| 14 | 30 | 1 CFU/278 g |
| 15 | 60 | 1 CFU/526 g |

[a] Assumed standard deviation of population within the lot is 0.8.

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When quantitative data are available, 3-class attribute sampling plans can be used if three states related to microbiological safety or quality can be differentiated: acceptable, unacceptable, and marginally acceptable (Figure 5). The concept of marginally acceptable deals more directly with the distribution of a microbiological concern within a food and consideration related to process control. It introduces a manufacturer's concern that not only should a lot not exceed a pre-established microbiological criterion, but also that the number of sampling units approaching that limit should not be too great. Four parameters must be established to describe a 3-class sampling. The term *n*, the number of sample units analyzed, is the same as described above for 2-class plans. The term *M* is used to specify the level above which the lot will be found to be deemed unacceptable if present in any of the sample units tested. The term *m* specifies the level of the microbiological contaminant above which (but below *M*) a sample unit is deemed to be marginally acceptable. Finally, *c* is the number of sample units that can be marginally acceptable and still consider the lot as acceptable. No equivalent *c* value is specified for *M* since by definition the *c* for *M* is zero.

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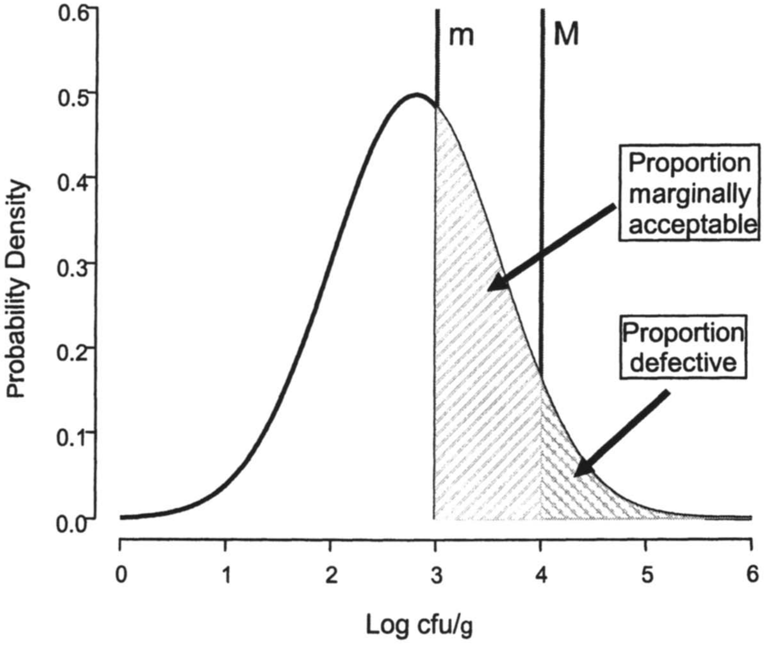

***Figure 5. Graphical depiction of a 3-class attribute sampling plan and its decision criterion for unacceptable (M) and marginally acceptable (m) sample units. (Adapted from ICMSF, 2002)***

As with 2-class plans, the discriminatory power of 3-class sampling plans is dependent on the number of samples (*n*) examined, the value of *c*, and the size of the analytical unit examined. However, the calculation of acceptance probabilities is more complex because there are two underlying defective rates being captured, the % defective for sample units exceeding *M* and the % defective for sample units exceeding *m*. Thus, the discriminatory power of 3-class sampling is also dependent on the differential between *m* and *M* values. Care must be taken in selecting the *m* and *M* values. Ideally, the *M* value is set based on a consideration of the risk associated with a microbiological hazard. On the other hand, the m value is based on the level of control that is expected and reflects the performance of a food safety or food quality control system when it is operating under control. Thus, exceeding the *m* value is a reflection of the distribution within the lot being different from what was expected as a result of either the mean log value of the hazard increasing and/or the variance of the population of sample units within the lot as compared to a pre-established criterion.

One of the areas related to microbiological testing that is often misunderstood is the use of indicator/index microorganisms (*2, 4*). These involve the detection of surrogate microorganisms. Indicators are microorganisms (or their products) that are indicative of a condition that is associated with a microbiological safety concern, such as the detection of *Escherichia coli* biotype 1 as an indicator of fecal contamination. Alternatively, index microorganisms are those whose presence is indicative of the co-presence of specific pathogen (*e.g., Enterobacteriaceae* as an index of *Salmonella* contamination). It is very important the basic assumptions and limits underlying an indicator or index assay be fully understood initiating such a program. For example, while *E. coli* is well recognized as an indicator of fecal contamination, in refrigerated foods its levels may be more indicative of the extent of storage at abuse temperatures than the actual level of initial fecal contamination (*4*). It is also important to note that for index microorganisms that the correlation between the surrogate and the pathogen often are not strong at low levels of contamination.

## Between-Lot Testing

While the within batch testing of lots to make decisions about safety have a long history of use, it has long been realized that safety and quality need to be designed into a food and not based on inspection after the fact. Thus, the past 40 years has increasingly seen the adoption of food safety systems such as HACCP. However, an integral part of any food safety control system is the periodic verification that the system is achieving its goals. This has increasingly led to the use of microbiological testing for a different manner, the evaluation of process

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control. In this application, the goal is not to establish that any single lot of food is safe or meets some specified level of quality. This is assumed to be achieved by the design of the food control system. Instead, the purpose of this type of testing is to verify that all or part of a food control system is consistently functioning within design parameters.

The general approach is to take, over time, sample units at specified locations within the food chain. These samples are then arrayed as a function of time and examined for shifts from the normal distribution of results that would be expected. An important aspect of this testing is that no single lot is examined in detail. Instead, an underlying assumption in type of testing is that when the food control system is "under control" the variability from lot-to-lot is constant and reasonably small. Thus, when a larger than expected number of defective units are detected within a specified time period it can be interpreted as a loss of control leading to an increase in either the mean concentration of a microbiological hazard or an increase in the processes variability (*2*).

Plotting microbiological data over time, which is commonly referred to as "control charting," can be extremely instructive. When a food control system is functioning as intended, quantitative measurements of the microbiological concern being controlled will typically cluster around a central value and have a characteristic variability (*2, 3*). Even when a system is functioning as designed, it can be expected that a certain number of sample units will exceed acceptance criteria. However, such deviations are within the design parameter of the control system. When control is lost, the number of unacceptable sample units within a specified time period begins to exceed the design specifications. The type of problem that is occurring is immediately evident by examining the changes over time. For example, Buchanan (*2*) provided hypothetical examples of the changes in control charts that would be expected for different classes of process failures within a microbiological control system (Figure 6).

Effective use of between lot microbiological testing in conjunction with process control statistics techniques requires that the user have detailed knowledge of the food control system they are evaluating. This generally requires that a substantial amount of data be collected initially to determine the characteristics of the system when it is operating under control and the variability inherent in the system. This may require the collection of data over the course of a year if there is substantial seasonal variation. However, an interim evaluation of the system can be undertaken during its initial operation, interim performance criteria established based on those data, and the values can be further refined as more data are collected during the normal operation of the microbiological testing program. In many instances the control limits established for a process control are based on the variance of the control system. For example, the microbiological population present after the manufacture of a food is log normally distributed, then when converted to log numbers, 99% of the

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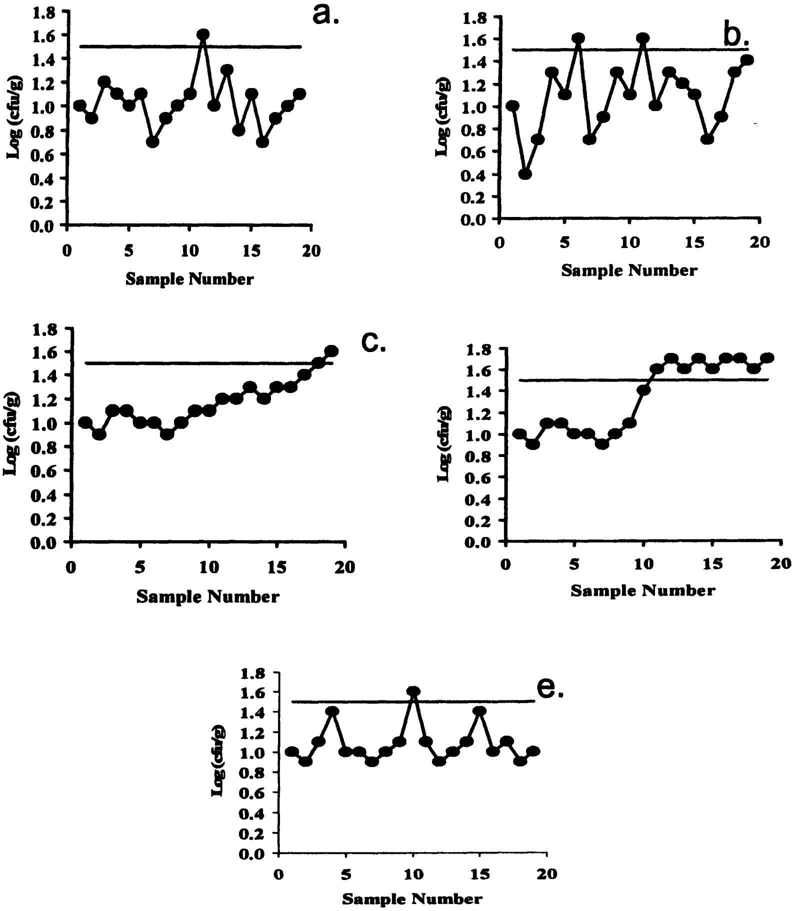

*Figure 6. Hypothetical examples of using control charting to relate the loss of control of a food control system and the cause of the failure: (a) system operating under control, (b) loss of control due to excess variability (i.e., uncontrolled step in process that contributes substantially to overall variability), (c) loss of control due to a gradual failure of a control step, (d) loss of control due to an abrupt failure of a control step, and (e) loss of control due to a reoccurring, transitory failure of a control step. Solid horizontal line depicts a hypothetical microbiological criterion above which a sample unit is only considered unacceptable. A criterion based on the presence of more than a specified number of unacceptable sample units within a specified period of time or sequential sample units would then be the basis for determining if a process is out of control and requires corrective action. (Adapted from Buchanan, 2000)*

values should fall within 3σ (3 standard deviations) of the mean. If >1% of the sample units fell outside that range it would be indicative that the system was no longer operating as originally designed. Conversely, this approach can be used to estimate the frequency at which values exceeding a specified number of standard deviations (e.g., >3σ) occur and the system is in control. For example, control charting in combination with a probabilistic model was used to predict the frequency of unusually elevated bacterial counts that would be expected to occur in a food (*5*, *6*).

As with within-lot microbiological testing, between-lot testing can employ both variables (quantitative data) and attribute (presence/absence data or binned quantitative data) techniques. Among the simplest, widely-used form of within-lot testing is the moving window sum. This type of attribute-based process control testing is the basis of process control verification microbiological testing programs established in conjunction with the USDA Meat and Poultry HACCP Regulation (*7*) and the FDA Juice HACCP Regulation (*8*). Moving window sampling plans involve performing and control charting microbiological analyses for the presence/absence of a pathogen or surrogate microorganism over time. A "window" is established, consisting of a specified number of analyses (*n*) starting from the most recent. As a new result is acquired the window "moves" by one to accommodate the most recent result and drop the oldest value. The second parameter defined in a moving window sum is the *p* value or probability that the microorganism of concern is present. The *p* value is expressed as the number of analyses that can be positive (i.e., presence of the microorganism) and the process still be deemed as under control. This is equivalent to the *c* value in within-lot attribute testing.

As with 2-class within-lot attribute plans, the operating characteristics of a moving window sum plan is dependent on the *n* and *p* values selected, and as such have strengths and weaknesses as related to the percent of defective samples occurring with a population of sample units being produced over time. Techniques such as the moving window sum are extremely sensitive to major changes in the rate of defective sample units being produced. A small window (i.e., *n* value) will rapidly detect a major shift in *p* but may miss a more gradual shift. Conversely, a large *n* value is more discriminating, and will detect small changes in *p*, but delay the discovery of the shift. It may be possible to overcome this need for short term responsiveness for large shifts in control versus the ability to detect small shifts in control by simultaneously charting the results against two windows, one long and one short. A similar problem arises after a problem has been identified and needed corrections are made. A small window allows the rapid "resetting" of the window to the "under control" state, but lacks the sensitivity to verify control of small differences in defect rates. An alternative in this situation is to perform intensive sampling over a shorter time frame.

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Between-lot attribute testing can also be used in conjunction with binned quantitative data, and has advantages over variables testing if concern is only whether a specified value is exceeded. However, in most instances where quantitative data are being acquired one should consider the use of a variables between-lot testing technique. This type of testing is most often used in conjunction with physical measurements associated with processes controlling the microbiological safety or quality of a food, however, the techniques could be adapted to use with microbiological concentration data. For example, Murphy et al. (*9*) used a variables technique to evaluate the level of process control afforded by a thermal treatment of chicken leg quarters and then validated the treatment as capable of consistently being able to achieve a 7-log inactivation of *Listeria monocytogenes*. It should be noted that there are unique limitations associated with the use of variables techniques if a substantial portion of the sample analyses result in non-detection of the microorganism of interest. There are a variety of process control variables techniques that are available and standard references are available that describe their characteristics and applications (*10, 11*). In this instance it is to differentiate whether the "zero" values are due to the microorganism not being present or it is present at a level below the lower limit of sensitivity for the methods. As a general rule of thumb, if more than half of the analyses are non-detection of the microorganism of interest, between-lot attribute techniques are likely to be more appropriate.

The effective use of microbiological testing techniques is an integral part of any integrated food safety control program. It is critical for validating that the control program is effective, verifying the programs are operating as intended, investigating and correcting problems when they arise, and deciding the safety and quality of foods when no other knowledge is available. Each of these uses requires different approaches and tools. Regretfully, too often there is incomplete understanding of the issues related to the acquisition of samples, the sensitivity of the methods, and the statistical underpinnings of both. Without this understanding, these potentially powerful tools for helping to assure the microbiological safety and quality of the food supply cannot deliver the information they are intended to provide. Thus, an integral part of the training of food microbiologists must be a firm understanding of the statistical concepts and techniques as applied to microbiological methods and sampling plans.

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## Chapter 14

# Predicting the Growth of Microbial Pathogens in Food

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Predictive microbiology has emerged as an important field of applied science that describes the growth, survival and inactivation of microbial pathogens through mathematical expressions. Predictive models are especially useful for estimating responses of pathogens to intrinsic and extrinsic environmental factors that have not been experimentally tested. They are widely used to design and implement Hazard Analysis and Critical Control Points food safety systems, including identifying Critical Control Points, associated Critical Limits, and potential remedial actions when process deviations occur. Models are most valuable when they have been validated for specific pathogen-food combinations, and accepted by regulatory agencies for making food safety decision. This chapter discusses key steps in the design, production, and validation of pathogen growth models.

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Microbial pathogens can display three primary patterns of behavior in food: growth, an increase in viability; survival, no significant change in cell numbers; or death, a decrease in viability. The science of predictive microbiology is based on the premise that such microbial behavior can be describe by mathematical expressions and that it is reproducible for a specific set of environmental conditions. An additional assumption is that changes in behavioral parameters form smooth surfaces, allowing for predictions over interpolative regions that have not been experimentally tested.

Validated microbial models have proven to be valuable tools for risk managers in food companies and pubic health organizations, in that they reduce uncertainty about estimations of risk. Models are actively used to develop and implement Hazard Analysis & Critical Control Points (HACCP) food safety systems and to estimate human exposure in quantitative microbial risk assessment. As such, their value to risk managers continues to increase, and drives new research leading to the development of more accurate and robust models.

This chapter provides the reader with practical discussions about the design, production, and validation of models for predicting the ***growth*** of microbial pathogens in food. For perspectives on thermal and non-thermal inactivation modeling, as well as general concepts and applications of predictive microbiology, the author recommends articles by Juneja (*1*), McKellar and Xu (*2*), McMeekin et al. (*3*), and Ross and McMeekin (*4*).

## Pathogen Growth in Food

Bacterial pathogens typically display up to three different phases of behavior in food: lag, growth, and maximum population density (i.e., stationary phase).[1] These phases can be defined by fitting "primary" curves to the kinetic data, and are commonly referred to as growth parameters. Lag phase duration (LPD) is normally expressed in units of hour or day; growth rate as the log of cell counts per hour or day; and maximum population density (MPD) as counts per gram or milliliter of the matrix.

For a specific set of environmental conditions, repeated experimental testing shows that growth rate and maximum population density vary less than lag phase duration (LPD). As described in greater detail below, LPD is not only dictated by the innate properties of the cell, but also by the cell's previous "history," more specifically its physiological state before it has transferred to a new environment (*5*).

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[1] In some instances, a death phase may occur, however this chapter focuses on growth scenarios only.

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The change in a primary growth parameter as a result of environment condition is described by secondary models or response surfaces. These surfaces have interpolative regions which encompass the experimental range of independent variables, and where model predictions have greater accuracy. Predictions out of this region are termed extrapolations, and are inherently uncertain.

The literature is replete with growth models based on the behavior of single bacterial cultures in defined microbiological media. Far fewer models have been developed for marketplace foods, and less so for non-sterile foods where pathogens and native flora compete for survival. As a result, our perspectives on bacterial behavior are skewed towards homogeneous environments where single microbial species grow at fast rates and to high densities. It can be argued that models based on these defined test systems allow one to more clearly isolate the effect(s) of individual variables. In addition, such models generally provide more liberal estimates of bacterial growth, referred to as "worst-case" predictions. However, it can also be argued that models produced from pure cultures in defined media do not adequately fulfill the needs of risk assessors and risk managers who are interested in knowing realistic levels to which humans are exposed. Also, such liberal growth estimates may lead to over-designing food processing operations that expend unnecessary capital to control pathogens, while also reducing the quality of foods.

## Growth Rate

Factors that influence bacterial growth rate in food include intrinsic factors such as nutrient level, pH, water activity, and acidulants. Extrinsic factors are commonly temperature, the type of gaseous atmosphere, and relative humidity. In addition, growth rate can be influenced by the presence of native microbial populations (e.g. spoilage organisms), especially when the latter are present at proportionally higher levels than the pathogen, and at refrigeration temperatures where spoilage organisms typically have higher growth rates.

In general, there is a positive relationship between temperature and growth rate, and an inverse relationship between temperature and the generation or doubling time. In the field of predictive microbiology, growth rate is expressed as the change in cell number per unit time. In mathematical expressions, growth rate is normally expressed in natural logarithm (ln) form, termed the "specific growth rate" ($h^{-1}$). One can convert the $\log_{10}$ form of growth rate to specific growth rate by multiplying the former by the ln (10) or ~2.303.

Figure 1 depicts the growth of *Listeria monocytogenes* on a slice of sterile cured ham at 37°C (*6*). The regions of the curve representing the three growth parameters (i.e., LPD, growth rate, and MPD) are illustrated with straight lines.

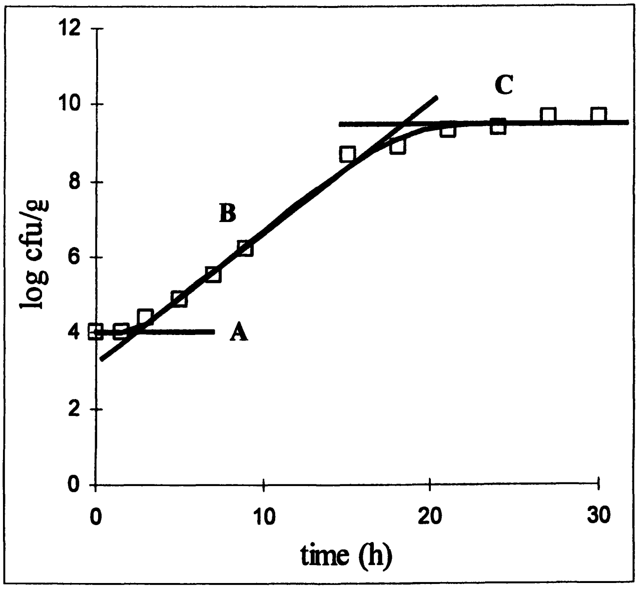

*Figure 1. Three parameters of bacterial growth: LPD (A), growth rate (B) and MPD (C).*

Figure 2 presents secondary model data and shows the observed growth rate ($\log_{10}$ colony-forming units (cfu)/h) for *Listeria monocytogenes* on a sterile cured ham product over a storage temperature range of -1 to 42°C (*6*). The rate steadily increases from low to high temperature, until reaching a maximum at 35°C. Beyond this temperature, growth rate decreases until *L. monocytogenes* reaches the no-growth boundary at approximately 45°C.

## Lag Phase

The LPD represents the time required for a bacterial cell, or cell population, to adjust to a new environment before cell division can occur. Baranyi and Roberts (*5*) refer to this time as "work-to-be-done." The dynamic growth model described by these authors, contains a term, $h_0$, which represents the

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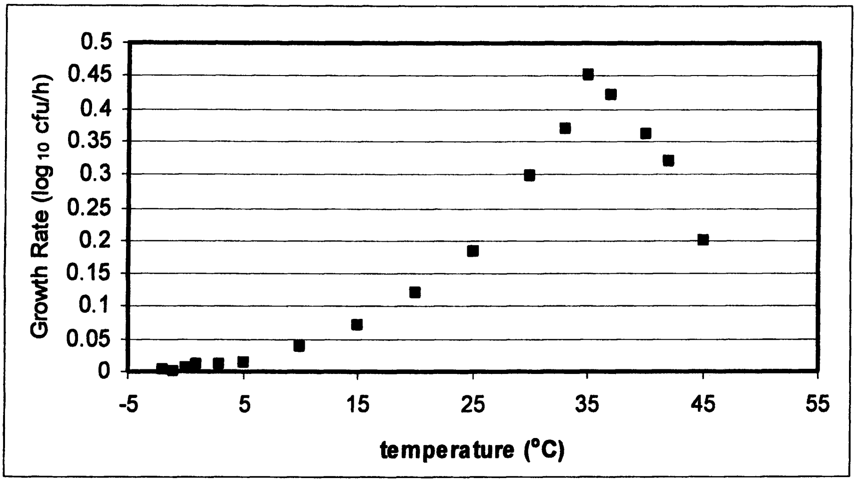

*Figure 2. Growth rate ($log_{10}$ cfu/h) of Listeria monocytogenes on sliced sterile cured ham as a function of incubation temperature (°C). The ham slices were inoculated by growing L. monocytogenes in Brain Heart Infusion broth at 37°C until late stationary phase, diluting the culture to ~3-4 log cfu/ml in 0.1% peptone water, and adding 100 µl to the ham surface. Each slice was vacuum-packaged and stored at -1 to 45°C.*

$$h_0 = \ln\left(1 + \frac{1}{q_0}\right) = \mu_{\max}\lambda \tag{1}$$

physiological adjustment that is related to the physiological state of the cells ($q_0$), and to LPD ($\lambda$) and growth rate ($\mu_{max}$).

In general, LPD increases with decreasing temperature, with possible exceptions near growth/no-growth boundaries. Much like growth rate, this temperature dependency is likely related to the thermodynamics of cellular adjustments (e.g. enzymatic reactions) that must occur before cell division. Figure 3 shows changes in *Escherichia coli* O157:H7 LPD over a temperature range of 10 to 40°C, in both sterile raw ground beef and Brain Heart Infusion (BHI) broth (*8*). The latter values were generated from a BHI-based model and show higher LPD compared to observations in sterile raw ground beef (*7*). Unlike in BHI, the LPD for *E. coli* O157:H7 in ground beef abruptly shifts from approximately 17 hours at 11°C to an undetectable LPD at 10°C. This paradigm demonstrates that broth models cannot universally be considered to safe when

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applied to foods, and this situation underlines why models must be validated when they are applied to different matrices.

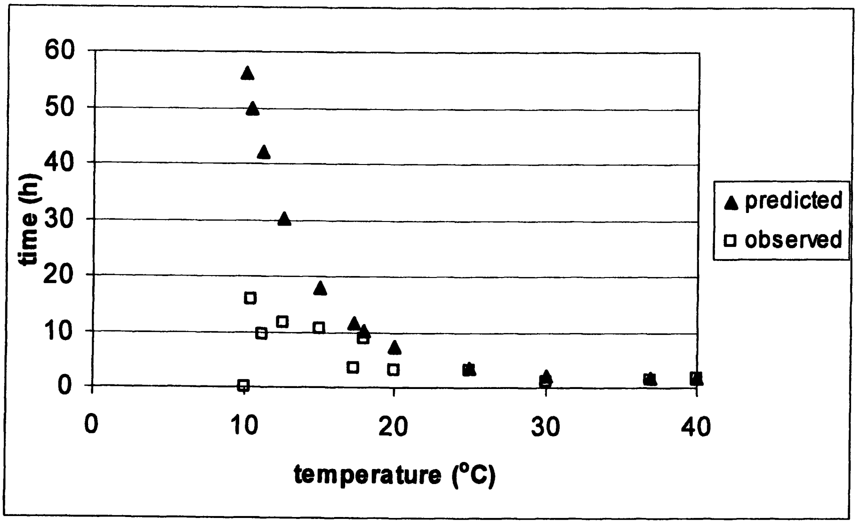

*Figure 3. Lag phase duration (h) of Escherichia coli O157:H7 in sterile raw ground beef as a function of incubation temperature (°C). Lag phase predictions based on a broth-based model (▲) are compared to observations in sterile raw ground beef (□). The ground beef was inoculated by growing E. coli O157:H7 in Brain Heart Infusion (BHI) broth at 37°C until late stationary phase, diluting the culture to ~3-4 log cfu/ml in 0.1% peptone water, and inoculating 90 g of ground beef with 10 ml of the diluted culture. Samples were placed in loosely sealed plastic bags and stored at temperatures ranging from 10 to 40°C (7). The E. coli O157:H7 broth model was developed from E. coli O157:H7 growth in BHI broth (8).*

Studies show that LPD increases when bacteria are "stressed," such as following heat- or acid-shock, desiccation, freezing, and when bacteria are exposed to relatively large shifts in temperature (*10*). However, one cannot assume that all environmental changes result in a lag phase. For example, a low or non-detectable LPD may not occur when bacteria are grown to exponential growth phase in food and then transferred to a similar food matrix. This likely occurs because the bacteria already have the necessary cellular constituents to commence growth in the new environment. The example shown in Figure 3 is a different example of this concept, in that *E. coli* O157:H7 cells grown to stationary phase in 37°C BHI broth apparently have the appropriate physiological state to immediately commence growth in 10°C sterile raw ground beef (*11*).

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Some may argue that there is relatively little value in modeling LPD due to its inherent high uncertainty. However, not to address this problem seems short-sighted considering the high demand for ready-to-eat (RTE) food products and the associated impact of *L. monocytogenes* on these foods. In this regard, numerous food companies are now incorporating additives, such as lactate and diacetate salts into RTE products in an effort to meet the new Food Safety & Inspection Service's regulation for controlling *L. monocytogenes* growth in RTE products (*12*). Furthermore, a company producing blends of lactates and diacetates has produced a time-to-growth *L. monocytogenes* model (i.e., a probabilistic LPD model) to predict shelf-life (www.purac.com). Baranyi (9) has also described a stochastic approach to modeling LPD.

Even though LPD is dependent on the previous history of cells, bacteria appear to display an innate distribution of LPD which could be useful for estimating LPD in the absence of knowing the cellular physiological state. Ross (*13*) reported that LPD is inversely related to the generation time (GT), and that the ratio of LPD to GT, referred to as "relative lag" (RL), represents a distribution of RL among independent data sets. Commonly, the distribution of RL for a particular species displays a mode in the range of 3 to 6. Thus, when knowing a species growth rate, it may be possible to predict a range of LPD at certain probability levels, thus reducing prediction uncertainty.

In recent years, more attention has been focused on defining factors that contribute to LPD, at both the single cell and population levels. Using a flow chamber where the replication of thousands of individual cells could be measured, Métris and coworkers (*14, 15*) reported that there is a distribution of LPD among individual cells. Furthermore, they show that the division time (DT) progressively decreases from the first, second, third and fourth DT. Such information will be useful in risk assessment to estimate pathogen outgrowth at low levels of contamination.

## Maximum Population Density

Maximum population density (MPD), sometimes referred to as "stationary phase" or the "carrying capacity" of the environment, denotes the highest concentration of bacteria that is achieved for a specific environmental condition. In single culture systems, this density is typically in the range of $10^8$ to $10^{10}$ log cfu per gram or milliliter of matrix (Figure 1).

When multiple species occupy an environment, the behavior of individual species is influenced by other members of the population. These interactions can result in competition for nutrients, inhibition by exogenous factors such as toxic metabolites, low pH, and the depletion of essential growth factors (*18*). The typical first approach to studying microbial interactions involves a test system of

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two species (*19*). Such a system facilitates quantification and dissection of microbial interactions, particularly the mediator(s) of the inhibitory effect.

In this regard, Figure 4 describes the MPD and growth rate of *E. coli* O157:H7 in the presence of *Hafnia alvei* in raw sterile ground beef at 10°C (*6*). Growth rate and MPD were measured for different ratios of *E. coli* O157:H7 and *H. alvei*, ranging from $10^4$:$10^0$ *H. alvei*:*E. coli* O157:H7 to $10^2$:$10^0$ *E. coli* O157:H7:*H. alvei*. In this test system, *H. alvei* exerted an inhibitory effect on *E. coli* O157:H7 MPD and growth rate, but not the converse. The *E. coli* O157:H7 MPD was reduced at ratios less than 2 logs, and likewise the growth rate at ratios less than -3 logs. At the latter ratio, *E. coli* O157:H7 growth was essentially halted.

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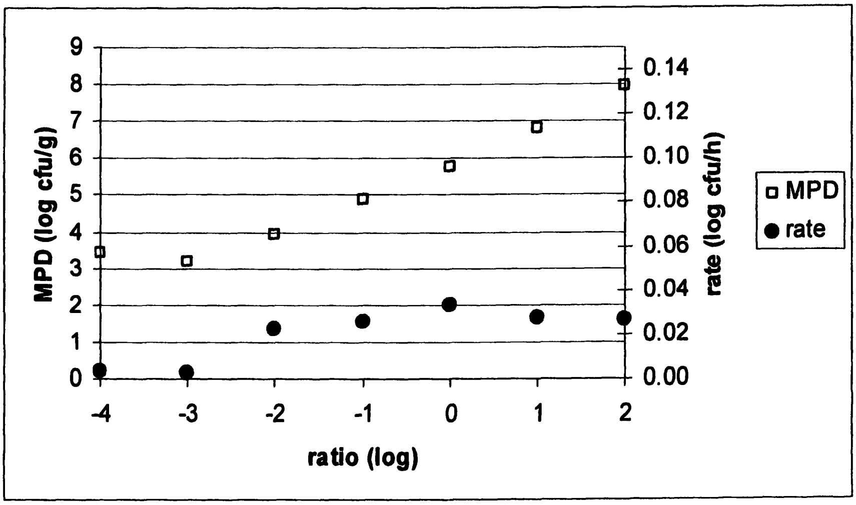

*Figure 4. Maximum population density (□) and growth rate (●) of Escherichia coli O157:H7 in 10°C raw ground beef at different inoculum ratios (log) of E coli O157:H7 to H. alvei. E. coli O157:H7 and H. alvei were grown in BHI broth at 37 and 30°C, respectively, to late stationary phase, diluted to in 0.1% peptone water, and added to 90 g of ground beef in a total of 10 ml. Samples were placed in loosely sealed plastic bags and stored at 10°C (6).*

## Experimental Protocols and Model Development

Predictive models represent mathematical expressions of the effects of environmental conditions on bacterial behavior and are useful for estimating pathogen behavior under conditions that have not been experimentally tested.

Primary models describe changes in microbial levels as a function of time, while secondary models estimate changes in parameters of microbial growth as a result of environmental conditions. Tertiary models, the interface between the model and the user, predict growth within the interpolative range of experimental conditions.

Predictive models are classified as kinetic or stochastic. Kinetic models estimate changes in microbial numbers over time and stochastic (probabilistic) models predict the probability that an event will occur. Stochastic models are used when microbial behavior is more random, such as at the growth/no-growth interface.

Producing a model with satisfactory accuracy involves a series of experimental procedures. These include designing experimental protocols; producing primary, secondary, and tertiary models; and measuring model performance.

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## Experimental Design

One of the most important aspects in producing a high quality model is the experimental protocol. For the specific pathogen-food combination of interest, the protocol must include all relevant variables that are expected to influence the growth of the pathogen. In situations where one or two variables will be modeled, a full factorial experimental design may be appropriate. When three or more variables are to be modeled, then full factorial experimental designs become unwieldy, and a partial factorial design is prudent.

If one wishes to predict the growth of a pathogen in food, then it is best to use strains that have been isolated from food and/or food processing environments. Clinical strains may have mutated during human passage, with possible changes to primary growth parameters. Also, strains that have been maintained in frozen storage are preferable because they retain relevant characteristics that may be lost during repeatedly subculture.

The microbial composition of foods can have profound effects on the growth of pathogens. If the user desires a model with worst-case scenario predictions, then a sterile food may be more appropriate, or one that has a lower level of endogenous flora. Another option is to inoculate the test matrix with only the pathogen and one or two other strains, thus facilitating pathogen enumeration, and isolation and measurements of the inhibitory substance(s).

If lag phase predictions is an important goal of the research, then the experimental design must include various preparations of the pathogen that closely represent the anticipated physiological states of the pathogen before it contaminates food. For example, the inoculum may need to be heat- or acid-shocked, grown to a specific growth phase, desiccated, and/or exposed to nutrient-limiting environment. Such detailed considerations of the experimental protocol will ultimately result in more accurate models with broader applications and use by risk managers.

## Primary Models

After the experimental protocol is established, kinetic data are collected for each of the independent variable conditions. Next, curves are fit to the data to describe growth parameters: LPD, growth rate and MPD. In instances where probability-of-growth data is relevant, data may be scored simply as growth (1) or no-growth (0).

Historically, the Gompertz model was one of the first mathematical expressions used to describe sigmoid-shaped bacterial growth curves (*20*). More recently, the dynamic model described by Baranyi and Roberts (*5*) has gained widespread use in predictive microbiology research, due to its mechanistically-based parameter for LPD and its ability to predict growth under dynamic conditions, such as fluctuating temperature (16,17). The model is:

$$\frac{dx}{dt} = \frac{q(t)}{q(t)+1} \cdot \mu_{\max} \cdot \left(1 - \left(\frac{x(t)}{x_{\max}}\right)^{m}\right) x(t) \qquad (2)$$

where $x$ is the cell number at time $t$, $q(t)$ is the concentration of a limiting substrate, and $x_{\max}$ is the MPD. Other primary growth models are discussed in detail by McKellar and Xu (*15*).

## Secondary Models

Secondary models predict changes in primary model parameters as a function of the environment. For example, LPD or MPD can be predicted as a function of temperature, NaCl level, water activity, and acidulants. Secondary models can be simple linear regressions or more complex polynomial models. The literature describes square-root, gamma and cardinal models for LPD and growth rate (*22*). The use of probability models for describing microbial growth is increasing. Applications include predicting pathogen time-to-growth, pathogen growth at the /no-growth interface, and toxin production. Ross and Dalgaard (*22*) provide a detailed examination of probabilistic modeling techniques and applications.

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## Tertiary Models

Secondary models, and their associated mathematical expressions, are often times too complex for ready adaptation by food industries and regulatory organizations. Consequently, it is helpful to design simple interfaces between the algorithms and input/output data. This is commonly done with spreadsheets, such as Excel, and with stand-alone programs designed with Visual Basic and .Net programming languages.

Currently, there are a number of free software packages that can be used to predict the behavior of pathogens and spoilage organisms, as well as food product shelf-life. The Agricultural Research Service's *Pathogen Modeling Program* (PMP; www.arserrc.gov/mfs/pathogen.htm) software package contains individual model interfaces for pathogen growth, survival, inactivation and toxin production in foods and microbiological broths.

*Growth Predictor*, produced by the U.K. Institute of Food Research (http://www.ifr.ac.uk/Safety/GrowthPredictor/default.html), is a stand-alone collection of models that predict the growth of pathogenic and spoilage organisms in microbiological broth. The Danish Institute for Fisheries Research http://www.dfu.min.dk/micro/sssp/Home/Home.aspx produces the *Seafood Spoilage and Safety Predictor* (*SSSP*) for predicting microbial spoilage of fishery products with fixed and changing temperatures, as well as the growth of *L. monocytogenes* in smoked salmon. Another software package that integrates with temperature data loggers is the Food Spoilage Predictor (http://www.arserrc.gov/cemmi/FSPsoftware.pdf) developed at the University of Tasmania, Australia.

## Measuring Model Performance

The utility of a microbial model for making food safety decisions is closely linked to its prediction accuracy. This assessment of model performance is commonly referred to as model *validation* or *error of calibration* when accuracy is measured against independent data, and model *verification* or *error of calibration* when predictions are compared to dependent data (i.e., the data used to produce the model).

Due to the increased use of predictive models by food companies and food safety regulators, the issue of what defines acceptable model performance is being debated (*23*). Comparing model predictions to observation is certainly not a new practice, in that the root mean square error (RMSE) test is well established. Baranyi *et al.* (*24*), Delignette-Muller *et al.* (*25*) and Ross (*26*) have described performance measures for predictive model bias ($B_f$) and accuracy ($A_f$), where $B_f$ is a mean measure of over- and under-prediction, and $A_f$ is an expression of cumulative model error. Equation 3 and 4 are based on the work of Ross (*26*) and are commonly reported in the literature.

$$B_f = 10^{\left(\Sigma \log(GT_{pred.} / GT_{obs.})\right)/n} \tag{3}$$

$$A_f = 10^{\left(\Sigma \left| \log(GT_{pred.} / GT_{obs.}) \right|\right)/n} \tag{4}$$

where *GT* is the generation or doubling time and *n* is the number of observations.

More recently, investigators describe a Robustness Index, representing the ratio of the standard error of prediction (SEP) to the standard error of calibration (SEC) for a given model, where the SEC is the RMSE of the growth model against the dependent data, and the SEP is the RMSE of the model against independent data (*27, 28, 29*). Such an index of predictive accuracy could be useful for defining the range of conditions for acceptable model application. In addition, the various sources of model error need to be described so that model users can know the extent of uncertainty that originating from experimental data, primary, secondary and/or tertiary models (*30*).

Predictive models are increasingly used in HACCP systems and risk assessment to estimate and control sources of foodborne hazards. They impact the development of policies that govern both national and international food commerce. As a consequence, predictive microbiologists need to develop more standardized and efficient approaches to model design, production, and validation. With such improvements, predictive models will reduce costs associated with food processing operations, facilitate the development of risk assessment, and lead to greater harmony in food safety regulations. Finally, the field of predictive microbiology will increasingly rely on multinational collaborations to produce, share and manage enormous amounts data. The US-UK ComBase (www.ComBase.cc) initiative is an example of progress in this direction, linking international researchers in a common goal of improving the safety and quality of the food supply (*31*).

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## Chapter 15

# Modeling the Behavior and Fate of Microbial Pathogens in Beef Processing Particle Reduction Operations

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Particle size reduction equipment, such as grinders and cutters, has wide application in food processes. Several studies have associated the particle reduction operation with product contamination by pathogenic organisms when processing meat. Models that describe the contamination and distribution of ground beef contaminated with pathogenic organisms, such as *Escherichia coli* O157:H7, have been developed for small and medium scale grinders as well as for a bowl-cutter. The contamination models describe the amount of ground beef contaminated as a function of the *E. coli* O157:H7 inoculated in the beef trims using linear and exponential models. The distribution models describe the contamination pattern as a function of the batch fraction processed in meat grinders and as a probability distribution function in a bowl-cutter. The concentration of pathogenic organisms was localized in selected components of the particle reduction equipment, thus deserving special attention from food processors when conducting sanitation operations.

In Advances in Microbial Food Safety; Juneja, V., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

There is a need for an understanding of how the particle reduction operation, conducted with equipment such as grinders and cutters, in meat processing interacts with a contaminated trim processed in a grinder or cutter and affects the finished product. Epidemiological studies of foodborne illnesses caused by *E. coli* O157:H7 and *Salmonella* sp. showed that these illnesses are associated with contaminated ground beef (*1*, *2*). In two of these studies, the meat grinder was implicated in the dissemination of these pathogens in ground beef. Banatvala et al. (*1*) traced an *E. coli* O157:H7 infection outbreak in the Bethel, Connecticut area in 1994 to supermarket grinders and other cutting utensils. Roels et al. (*2*) indicated that inadequate cleaning and sanitation of the meat grinder in a butcher shop was associated with an outbreak of *Salmonella* serotype Typhimurium in which 158 persons were infected in Wisconsin during the 1994 Christmas holiday period. The purpose of this manuscript is to present studies that have been conducted on the grinding operation with the purpose of modeling the pathogen distribution, and product and equipment contamination, in particle reduction operations in meat processing.

## Particle Reduction in Meat Processing

Particle size reduction equipment, such as grinders and cutters, has wide application in food processes. Meat grinding is a process that combines particle reduction with the extrusion of fibrous materials. Considerable work has been done to understand and optimize the grinding of particulate and dried materials; however, many of the grinding principles used to determine particle size reduction are not consistent over different ranges of applications (*3*). Even though there has been a need to study the grinding behavior of fibrous materials, such as beef, under the unit operations approach (*4*), there is little information on this subject in the current literature. Studies conducted on meat grinding have investigated the design elements used for the removal of bone and non-meat materials for different meat species, different meat cuts, fat levels, and processing temperatures (*5*, *6*, *7*, *8*). In terms of the grinding operational parameters, the research has focused on the determination of (1) the plate pressure at the grinder die, and (2) the effect of grinding parameters (such as screw speed, feed diameter, and grinding plate hole diameter) on grinding energy, mass flow rate, torque and die configuration (*7*, *9*). Studies that had looked at the contamination in particle reduction of beef processing are limited and will be mentioned in the following paragraphs as the subject of each study is covered (*10*, *11*, *12*, *14*).

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# Modeling the Contamination with Pathogens in Particle Reduction

Modeling is important to engineers, food scientists and risk analysts because it provides for a mathematical representation of a process. The mathematical representation is a tool that could be used to understand a phenomenon and take appropriate measures to control a problem or limit its effect in a process.

Due to the impossibility to determine when a pathogen is going to be present in a food product, researchers have recurred to the artificial contamination of food batches with selected mutant pathogens to facilitate the pathogen detection and determination. Techniques used in the development of the mutant pathogen are described by Farrell et al. (*10*), Wei et al. (*15*) and an example of the study of the mutant is presented in Flores (*13*). Under this approach, meat is artificially inoculated with the mutant pathogen of interest, processed and the processed product and equipment is tested for the pathogen detection using the appropriate microbiological technique. Models are developed based on an experimental design targeting a measurable response, i.e. counts per unit of weight in the ground product, as a function of a set of variables such as inoculum level and batch processed.

Figure 1 shows a representation of a sample of data collected from contaminated ground beef processed in a mid-scale grinder (35 $gs^{-1}$) in which a beef trim was inoculated with a rifampacin resistant *E. coli* O157:H7 (*E. coli* O157:H7$^{rif}$) at two different levels and *Listeria monocytogenes* at one level. The first peaks appear when 20-25% of the beef was processed as a result of grinding the inoculated trim. From that point on the contamination in the ground product decreases somewhat uniformly with some tested segments not showing detection. Occasionally a peak is detected.

Meat grinders and bowl-cutter have been used to study the effect of particle size reduction on the fate of pathogens and their distribution in the finished product. From an operations point of view, meat grinders differ from bowl-cutters in that the first one is a continuous process in which the tissue is ground once in the grinder; while in the bowl-cutter the meat tissue is in a batch operation in which the material is reduced in a consecutive and repetitive manner. Studies conducted on these particle reduction units have led to contamination and distribution models for pathogens. Also, as part of these studies the residual contamination left in the equipment has been studied.

## Contamination Models

The purpose of contamination models is to estimate the volume of finished product that a given amount of pathogen contaminates. The number of ways a

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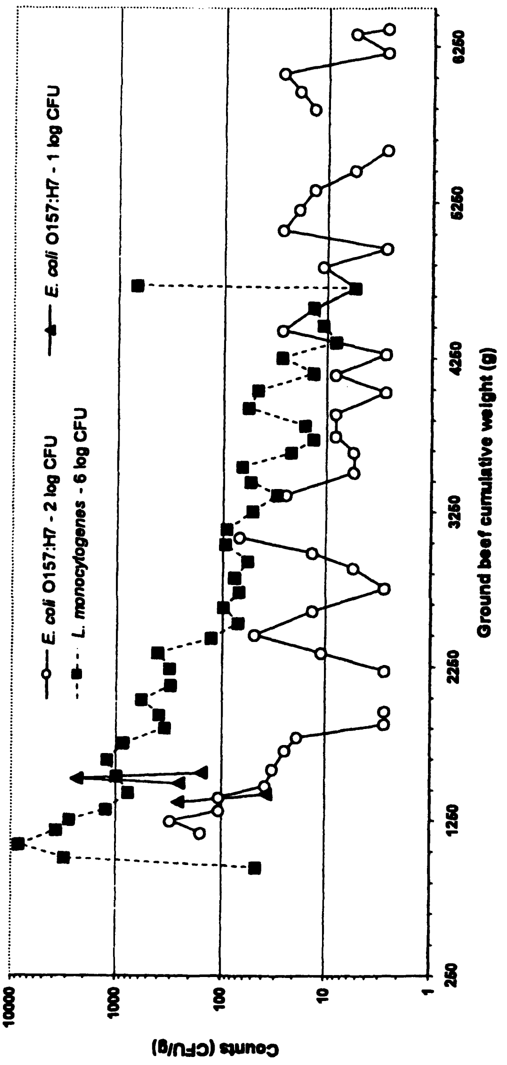

*Figure 1. Sample data collected from the ground beef processed in the mid-scale grinder*

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beef trim or batch of trims to be ground gets contaminated with pathogens is too large to test all alternatives experimentally. Thus, most studies add one contaminated trim with the mutant pathogen to a batch. Then, the batch is ground and the ground beef produced is tested for the mutant pathogen. In the present studies, two major assumptions were considered to define an amount of ground product as contaminated (*11*, *13*, *14*). First, the contamination is caused by a single colony of cells located on the surface of a beef trim that enters the grinder without any prior contact with other beef trims. Second, because the studies tested 100% of the beef batch processed in the size reduction equipment, the detection of one cell in a ground product sample defines that amount of sample as contaminated. In turn, the models developed were defined by algorithms in which the amount of contaminated ground beef is a function of the inoculum level added to the batch of beef trims.

Two contamination models for ground beef were developed for grinders of two sizes (*11*, *14*). The grinders used were a laboratory or small-scale grinder and a mid-scale grinder, with capacities of 6.5 and 35 $gs^{-1}$ for the small-scale and the mid-scale, respectively. The contamination model for the small-scale grinder was described by a linear model in which the amount of contaminated ground beef was a function of the inoculum level. The inoculum was placed on the surface of one beef trim and it was distributed over one square centimeter. The linear model was described by the following algorithm: $y = 101.8\ x$, where y is the amount of contaminated ground beef (g) and x is the inoculum level in log CFU. The model had a coefficient of determination of 0.933 (*11*). This model was validated with another sample inoculated with 4.1 log CFU of *E. coli* O157:$H7^{rif}$ to observe how close the model predicted an extrapolation of the small-scale model. The results for the amount of contaminated ground beef in the validation experiment were within the 95% confidence level interval of the model. For the mid-scale grinder the contamination model was better represented by a power model defined by $Y = 242.4\ X^{1.74}$, where Y is the amount of contaminated ground beef (g) and X is the inoculum level in log CFU. This power model had a coefficient of determination of 0.824 (*14*). The results of these models indicate that for the same amount of inoculum, the amount of ground beef contaminated increases with the size of the grinder.

## Distribution Models

Distribution models are defined herein as the mathematical algorithms that describe the allocation pattern of the pathogens in the finished product produced by the particle reduction equipment. Such a model assists in understanding contamination of the ground beef. Researchers have looked at single and multiple grinding to determine distribution models. Single, multiple and consecutive grinding operations are common in the meat industry. In many instances these are known as coarse and fine grinding. These operations take place one after the other to accomplish the desired texture and particle size in the product. The die openings could be of the same diameter or smaller for the fine grinding versus the coarse grinding.

### *Single grinding operations*

Flores and Stewart (*14*) determined distribution models capable of describing the level of *E. coli* O157:H7$^{rif}$ in the different fractions of ground beef as it leaves the grinder die (35 gs$^{-1}$). To develop their model, different algorithms were tested for each data set using TableCurve® 2D (*16*). For all the inoculum levels, the top three ranked models were defined by the following asymmetric peak functions: Chi-Squared, Lorentzian and Gauss-Lorentz cross product. The Chi-squared algorithm corresponds to an asymmetric peak function while the Lorentzian and Gauss-Lorentz cross product are symmetric peak functions. As shown in Figure 1, the pathogen distribution is not a symmetric function, thus the Chi-squared model was selected. Figure 2 shows the experimental and Chi-squared modeled results for two data sets, 3.2 and 2.1 $\log_{10}$ CFU inoculum levels of *E. coli* O157:H7$^{rif}$ in ground beef. The experimental results and the modeled data indicate that for a localized contamination on the surface of a beef trim ground, the pathogen distribution in the ground beef is not a random one but follows an asymmetric peak function. The Chi-squared algorithm had coefficients of determination that ranged from 0.814 to 0.999 for all the different inoculum levels tested (0 through 6 $\log_{10}$ CFU). Also, this model was validated using the results of additional grinding experiments with a 4.04 $\log_{10}$ CFU inoculum with a batch size equivalent to the batches processed to develop the model (7,000 ± 300 g). The results indicated a good fit with 0.85 coefficient of determination (*14*). Also, a coefficient of determination of 0.87 was found when this algorithm was tested using data for ground beef processed and contaminated with *E. coli* O157:H7$^{rif}$ in a small-scale grinder with a 2 $\log_{10}$ CFU inoculum level (*11*).

The distribution of the pathogens located on the surface of a meat trim processed into ground meat by a grinder is a physical phenomena controlled by the processing variables that affect mixing, conveying, extrusion, cutting and forming in the grinder. These variables depend on equipment design and power consumption by the operation. To evaluate this assertion, an experiment was conducted by the author in which instead of inoculating the beef trim with *E. coli* O157:H7$^{rif}$, fluorescent microspheres of 1.98 μm diameter were used as inoculum. The microspheres were added to the trim at a level of $1.3 \times 10^8$ units

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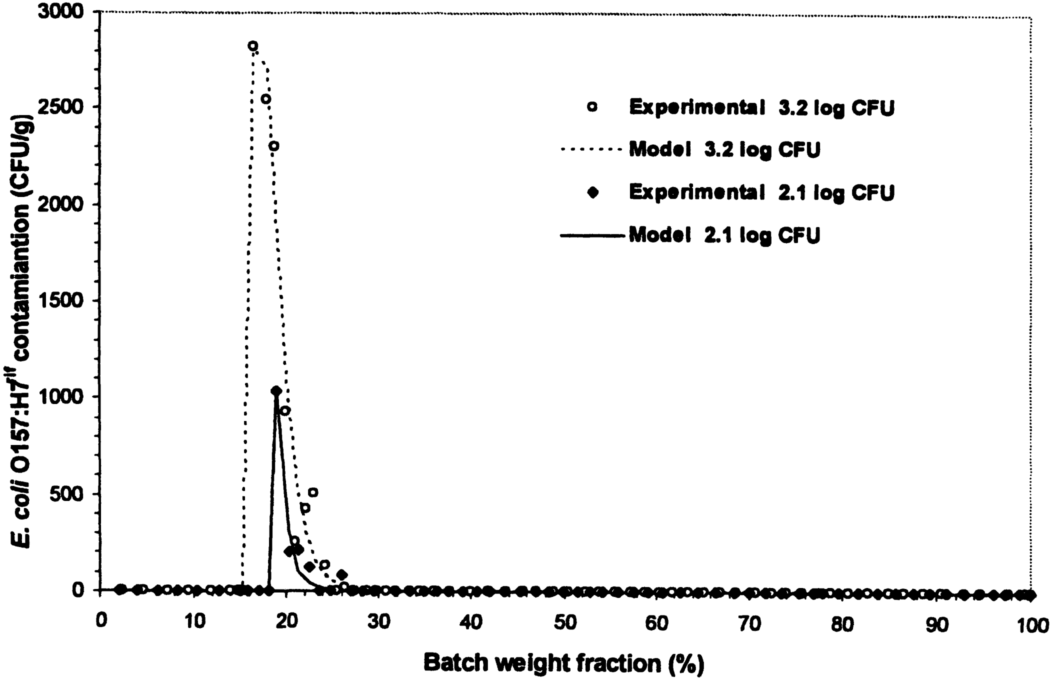

*Figure 2. Experimental and Chi-squared modeled results for the distribution of E. coli O157:H7$^{rif}$ in ground beef for two inoculum levels of 3.2 and 2.1 log$_{10}$ CFU.*

following the inoculation and grinding procedures indicated by Flores and Tamplin (*11*) for the largest inoculum. The microspheres were detected on the surface of the ground beef with the aid of a stereomicroscope and the image collected. This image was scanned with an image analysis software that transformed the presence of the fluorescent microspheres on the ground beef surface into pixels. The fluorescent pixels on the ground beef were plotted against the cumulative ground beef processed as well as the *E. coli* O157:H7$^{rif}$ detected in the ground beef for the 6.0 log CFU inoculum (figure 3). In Figure 3 the distribution of the microspheres overlaps the *E. coli* O157:H7$^{rif}$ detected on the ground beef, peaking at the point of insertion and following a similar distribution pattern. Figure 1 shows similar patterns to for *L. monocytones* and *E. coli* O157:H7$^{rif}$. These results led to conclusions that the distribution in ground meat of a localized pathogen colony on the surface of a meat trim is independent of the type of pathogen, unless the pathogens are firmly attached to the tissue.

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### *Multiple grinding operations*

As part of the grinding studies of the author, consecutive double-grinding experiments were conducted using the same equipment and operational conditions with the small- and mid-scale grinders. The material was ground once and then processed again in the same grinder. Figure 4 shows the pathogen distribution for the first and second grinding for the mid-scale grinder experiments for 3 $\log_{10}$ CFU inoculum level. When the ground beef was subjected to a second grinding, and the material was fed into the grinder in the same order as it was processed during the first grinding, the contamination with *E. coli* O157:H7$^{rif}$ was more uniformly distributed over the entire batch. Original large peaks were reduced (50 and 60% cumulative ground beef in the first grinding) and fractions that did show original contamination with *E. coli* O157:H7$^{rif}$ in the first grinding showed contamination in the second grinding (i.e. 15 and 35% cumulative ground beef in the first grinding). Thus, consecutive grinding tends to reduce the clusters by distributing the contamination more evenly.

Reinders et al. (*12*) studied the effect of up to three consecutive grinding processes in ground beef using a small-scale grinder like the one used by Flores and Tamplin (*11*). Reinders et al. (*12)* purpose was the determination of the random distribution or not of the microorganisms in ground beef. They conducted grinding experiments and looked at the variation in the endogenous flora of coliforms and artificial, clustered contamination of *E. coli* O157:H7. They arrived to the conclusion that grinding resulted in random and non-random distributed coliforms and *E. coli* O157:H7, respectively. Lognormal and Poisson (Gamma) distribution fitted the data, but only the latter one to determine if the microorganisms were or not randomly distributed.

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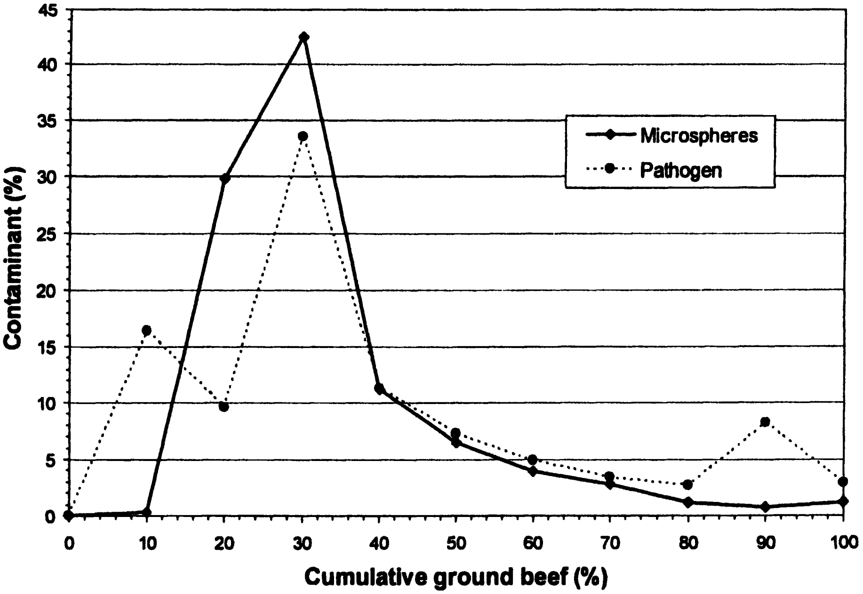

*Figure 3. Distribution of E. coli O157:H7$^{rif}$ and microspheres in ground beef.*

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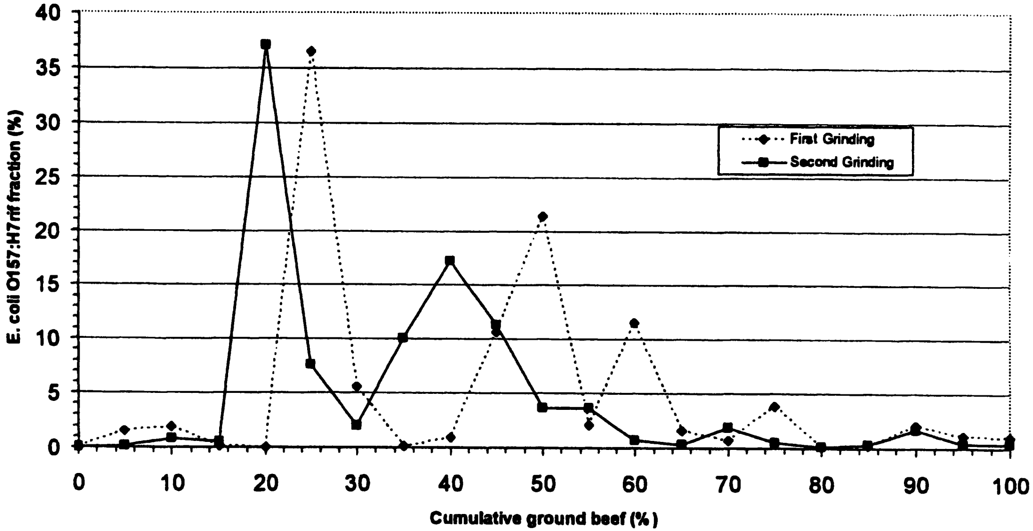

*Figure 4. Comparison of E. coli O157:H7$^{rif}$ distribution in ground beef when the batch is ground once and then a second time.*

An extreme case of multiple and continuous particle reduction operation is the bowl-cutter. In the bowl cutter the material is placed on a bowl and instead of passing through the blade and die, like in the grinder, goes through chopping blades many times depending on the rotation of the bowl. Flores (*13*) used a Hobart bench-top bowl-cutter (Model 84186U, Hobart Corp., Troy, OH) with a 45.2 cm bowl diameter rotating at 20 rpm and with two 8.8 cm blades rotating at 1720 rpm. To study the fate of the *E. coli* O157:H7 $^{rif}$ inoculated on a beef trim in a multiple particle reduction operation, such as the bowl-cutter; the ground beef in the bowl of the bowl-cutter was divided into twenty equal sections after grinding. All the material in each section was tested for the presence of *E. coli* O157:H7 $^{rif}$ to evaluate the distribution of the inoculum in the ground beef batch. For each experimental treatment, the average and standard deviation were calculated for the *E. coli* O157:H7$^{rif}$ detected in all the sections. Because the bowl-cutter is a particle reduction and mixing equipment, the average of the *E. coli* O157:H7$^{rif}$ detected for all the sections of one experiment indicates the distribution of *E. coli* O157:H7$^{rif}$ in the batch. In mixing studies, the standard deviation of the parameter measured, in this case *E. coli* O157:H7$^{rif}$ detected in the sections for each experiment, is the variability among the samples obtained in each batch (*17*). There were no significant differences ($P \geq 0.05$) among all the treatments for the averages and standard deviations of the counts of *E. coli* O157:H7$^{rif}$ distributed in the bowl samples. Thus, regardless of the different grinding times, batch size and feeding method, the *E. coli* O157:H7$^{rif}$ was distributed among the processed beef so that any section of ground beef in the bowl had enriched pathogen levels of 1.18 ± 0.27 log CFU/g (average ± one standard deviation). Figure 5 shows the experimental data with three probabilistic density functions: inverse Gauss, lognormal 2 and Pearson V. The probability function for the estimated *E. coli* O157:H7$^{rif}$ present in the ground beef processed in a bowl-cutter was best fit with the Pearson V function with the parameters depending on the size of the batch processed. This model corresponds to initial inoculum levels of 0.06 and 0.10 CFU/g for beef processed in 4 and 2 kg batches, respectively (*13*).

## Residual Contamination

Residual contamination left on the surfaces of meat processing equipment and plants is of major concern to food processors, government regulators and risk analysts. Considerable work has been done on attachment and viability of pathogens to different types of surfaces. However, the information about locations in specific processing equipment susceptible to potential storage of pathogens is limited. The following paragraphs describe the studies conducted to determine the potential pathogen reservoirs in particle reduction equipment.

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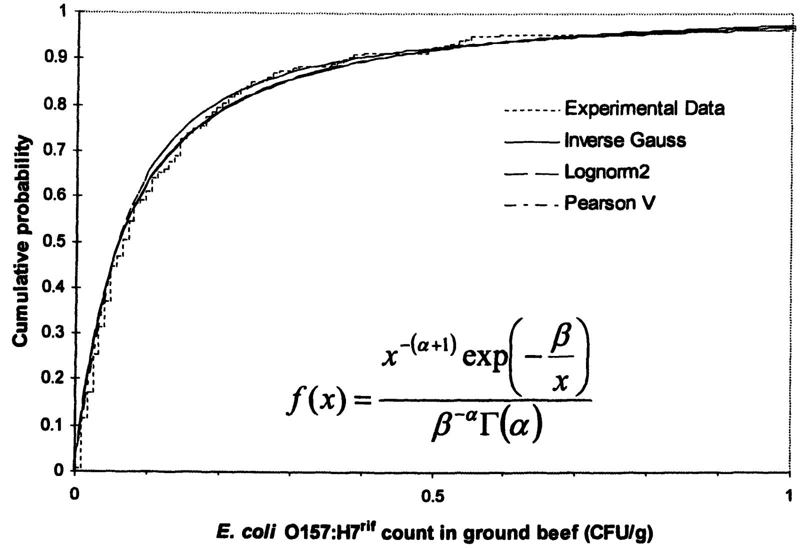

*Figure 5. Experimental results and cumulative probability functions for the E. coli O157:$H7^{rif}$ present in the ground beef processed in a bowl-cutter. Model shown corresponds to the Pearson V function (probabilities of 0.28 [$\alpha$= 2.90 and $\beta$=2.43E-02] and 0.75 [$\alpha$=1.83 and $\beta$=1.27E-02] for the 2 and 4 kg batches, respectively).*

Farrell et al. (*10*) looked at the attachment of *E. coli* O157:H7 to a meat grinder and pathogen survival after sanitation. They used 1- × 1-cm stainless steel chips glued to the sides and bottom of the auger housing of a meat grinder. After processing beef contaminated with a *E. coli* O157:H7 resistant to tetracycline and conducting sanitation operations, the residual contamination on the chips was detected. The inoculum levels they used were 6 and 2 log CFU/g. They detected different levels of residual *E. coli* O157:H7 on the chips depending on the sanitizer used and the sanitizer application procedure followed. However, their study only focused on the sides and bottom of the auger housing and did not look at other potential locations in the grinder.

Using a rifampacin resistant *E. coli* O157:H7, other studies previously referred (*11*, *13*, *14*) looked at all the parts in particle reduction equipment that could harbor pathogens after processing contaminated beef trims. Detection of *E. coli* O157:$H7^{rif}$ in the major components of a small-scale grinder indicated that the location that had the highest level of contamination was the grinder nut or collar that attached the die to the grinder housing and the second highest contamination was the grinder's screw (*11*). The following parts of a mid-scale

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grinder were evaluated: outside the die, grinder's collar, grinder's blade, meat left inside the screw and meatless screw (*14*). For the two highest inoculum treatments (5 and 6 $\log_{10}$ CFU) tested, all the points in the grinder tested showed contamination with *E. coli* O157:H7$^{rif}$. Also, for all treatments with inocula larger than 3 $\log_{10}$ CFU, the grinder collar was the location with the highest contamination. Residual contamination with *E. coli* O157:H7$^{rif}$ was found in the collar in all treatments with inoculums larger than 3 $\log_{10}$ CFU, but not with inoculums of 2 $\log_{10}$ CFU. Also, despite the trends at lower inoculum levels, *E. coli* O157:H7$^{rif}$ was detected for the 2 $\log_{10}$ CFU inoculum in the meat left in the grinder. The results obtained with the small-scale grinder (*11*) and the mid-scale grinder pointed at the collar or nut that attaches the die to the grinder housing as a location that harbors pathogenic contamination. The pathogen concentration in the collar could be due to the result of the combined action of the mechanical force of the screw compressing the meat against the blade and the centrifugal force of the blade spinning the low density materials, i.e. fluids carrying pathogens, toward the interior walls of the barrel or housing and to the grinder nut or collar. A large concentration of microspheres was detected in the grinder's collar in the experiment in which the *E. coli* O157:H7$^{rif}$ was substituted by fluorescent microspheres (Figure 3).

To test the plant practice of flushing the contamination with more material, Flores and Stewart (*14*) conducted extensive grinding experiments in the small and mid-scale grinders. Extensive grinding consisted of grinding an additional amount of material, 27% more beef added to the process. For the 2 and 3 $\log_{10}$ CFU inoculum treatments, no *E. coli* O157:H7$^{rif}$ was detected in the extensive grinding in the die, blade, screw or meat in the screw indicating that the *E. coli* O157:H7$^{rif}$ was apparently flushed from the system. However, for the 2 $\log_{10}$ CFU inoculum level, the contamination with *E. coli* O157:H7$^{rif}$ was detected in the collar that attaches the die and the blade to the grinder hub.

In the evaluation of the residual contamination in a multiple continuous particle reduction equipment, i.e. bowl-cutter, the pathogen distribution in the samples collected on the bowl-cutter's comb/knife guard and in the samples collected outside the bowl-cutter were not significantly different ($P \geq 0.05$) among all treatments (*13*). The level of contamination with *E. coli* O157:H7$^{rif}$ in the remnants varied and the highest level was not found in the same location. However, for all treatments, *E. coli* O157:H7$^{rif}$ was detected in the comb/knife guard when the *E. coli* O157:H7$^{rif}$ was present in the batch processed or if the batch was processed immediately after a contaminated batch. *E. coli* O157:H7$^{rif}$ was detected in the material that spilled outside the bowl-cutter in all treatments. The material that spilled outside the bowl was next to the knives and presumably spilled as a result of the cutting and grinding actions of the knives. After removal of all remnants, the surfaces of the bowl-cutter were swabbed and the counts detected were calculated based on the area swabbed by the sponge (64 $cm^2$).

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Flores' (*13*) results indicated that the distribution of the *E. coli* O157:H7$^{rif}$ on the equipment parts was a random process. Nonetheless, results show that *E. coli* O157:H7$^{rif}$ was not detected by swabbing the surfaces of the bowl-cutter components tested when operated for 60 s, while it was detected in all the other treatments with longer processing times (120 and 240 s). These results indicate that the longer the process, the more the *E. coli* O157:H7$^{rif}$ was distributed in the bowl-cutter components. Despite the fact that all the ground beef was removed from the bowl-cutter and an uncontaminated batch was processed after a contaminated batch in the bowl-cutter, *E. coli* O157:H7$^{rif}$ was detected on the bowl. After a second uncontaminated batch was processed following the one contaminated with *E. coli* O157:H7$^{rif}$, no contamination with *E. coli* O157:H7$^{rif}$ was detected in the ground beef, bowl-cutter components and ground beef remnants in the grinder. However, even though all the bowl-cutter surfaces were tested for *E. coli* O157:H7$^{rif}$ after the second uncontaminated batch, because the bowl-cutter was not completely rinsed, it should not be concluded that the *E. coli* O157:H7$^{rif}$ contamination had been removed from the system (*13*).

Particle reduction equipment in meat processing can work as a distributor of a localized surface pathogenic organism on meat. The particle reduction operation spreads a surface contamination over a larger portion of the batch and the equipment independently of the pathogen. The volume of contaminated finished product with the pathogen is dependent on the size of the localized contamination and follows a linear function for a small-scale grinder and an exponential function for a mid-scale grinder. The results of these models indicate that for the same amount of inoculum, the amount of ground beef contaminated increases with the scale of the grinder.

When the particle size reduction is conducted in a batch of multiple reductions, i.e. bowl-cutter, the entire batch gets contaminated and at least the immediate batch that is processed in the same equipment without a complete sanitation. Also, the pathogenic distribution that takes place in a meat grinder follows a pattern that is not random but follows an asymmetric peak function, such as the Chi-squared function. For multiple and consecutive grinder operations, the Pearson V function can describe the distribution of pathogenic organisms in the ground beef.

The collar that attaches the die to the grinder housing acts as a reservoir for pathogenic contamination. Thus, equipment designers and food processors should pay special attention while providing for easy removal of the collar and conducting sanitation operations on the grinder collar, respectively. In a bowl-cutter, the top of the comb/knife guard, and the knife are equipment surfaces most likely to retain the contamination. Therefore, periodical testing of the residues in the grinder collar, in any grinder size, and the top of the comb/knife guard and knife in a bowl-cutter would be a more appropriate measure for food

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processors to detect potential contamination in a batch of ground product than only random testing of the ground product.

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## Chapter 16

# Approaches for Modeling Thermal Inactivation of Foodborne Pathogens

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This chapter deals with some of the fundamental concepts concerning thermal processing of foods to eliminate foodborne pathogens. For most foodborne pathogens in a food matrix under isothermal conditions, the cell population generally decreases exponentially with heating time and, therefore, can be described by 1st-order kinetics. The resistance of microorganisms to heat is conventionally characterized by D and z values. For mixed cultures, if both D and z values follow 1st-order kinetics, a mixed-culture model can be used. With the latter model, the more heat-sensitive microorganisms will be preferentially inactivated, followed by the heat-resistant ones, explaining the "tail" effect observed in some survival curves. For more complex survival curves, a general "Weibull"-type model can be used. This model offers more flexibility in describing either convex, concave, or linear survival curves. This model is generally more accurate than the transitional linear model when used to describe convex and concave curves.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

## Kinetic Analysis of Bacterial Inactivation by Heat – Chemists' Approach

Like most chemical reactions, inactivation of bacteria is highly dependent on the time and temperature in the environment to which the bacteria are exposed. When a homogeneous population of a pure strain of bacteria is subjected to an environment maintained at a constant temperature, the reduction of bacterial counts generally follows 1st-order reaction kinetics (*1, 2*), which can be mathematically described by:

$$\frac{dC}{dt} = -kC. \tag{1}$$

In this equation, C usually represents the count of bacteria per unit mass or volume, t is the total heating time, and k is the rate constant. Under isothermal conditions, this equation can be integrated, resulting in:

$$\ln(C) = \ln(C_0) - kt \tag{2}$$

In eq 2, $C_0$ is the initial counts of bacteria. This equation reveals a log-linear relationship between the bacterial count and the heating time under an isothermal condition, indicating that the logarithm of bacterial counts decreases linearly with time as heating progresses. A plot of the logarithm of bacterial counts versus time is referred to as an inactivation or survival curve.

Equations 1 and 2 describe the change in the bacterial counts under an isothermal condition. However, the inactivation of microorganisms is highly dependent upon the heating temperature. The effect of temperature is usually manifested in the rate of inactivation during an isothermal process. Conventionally, the Arrhenius equation is used to described the relationship between the heating temperature and the rate of inactivation, such that:

$$k = Ae^{-\frac{E_a}{RT_K}} \tag{3}$$

In this equation, k is the rate of inactivation, A is a constant, also known as a frequency factor, $E_a$ is the activation energy (kJ), R is the gas constant $8.314 \times 10^{-3}$ kJ/mol K and $T_K$ is the absolute temperature (K).

The Arrhenius equation was originally developed by a Swedish chemist, Svante Arrhenius. He developed a collision theory to describe the reactions among molecules. He hypothesized that a chemical reaction between reacting

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chemical species was a result of their molecules colliding with each other. The activation energy, $E_a$, was the minimum energy required for the chemical reaction to occur. Increasing the reaction temperature would increase the kinetic energy of reacting molecules and, thus, increase the rate of reaction.

Equation 3 can be rewritten into a simpler form shown in eq 4. The new equation depicts a log-linear relationship between k and the inverse of T. If the logarithm of the rate is plotted against 1/T, a linear relationship should be observed. From the slope of the linear curve, the activation energy ($E_a$) can be determined. The frequency factor can be calculated from the intercept:

$$\ln(k) = \ln(A) - \frac{E_a}{R} \times \frac{1}{T_K} \tag{4}$$

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Thermal inactivation is generally believed to be caused by the irreversible denaturation of enzymes critical to support the biological activities of microorganisms. These enzymes are mostly complex large macromolecules distributed in the cells of microorganisms. At sufficiently high temperatures, these large protein molecules may be denatured and lose biological activity. Although eq 3 does not make any physical sense when it is applied to explain a physical process in a biological system such as inactivating microorganisms in foods, the 1st-order kinetics and the Arrhenius equation serve very well when used to describe the process of thermal inactivation of microorganisms in foods.

## Kinetic Analyses of Bacterial Inactivation by Heat – Food Scientists' Approach

Food scientists and food microbiologists have traditionally been using a different approach to quantify the presence of microorganisms in foods. Rather than using the natural log of bacterial counts, as shown in eq 2, they prefer using the base-10 logarithm of bacterial counts to quantify the number of microorganisms in foods. They also observe a log-linear relationship between the log counts of bacteria and the heating time under isothermal conditions (eq 5). Instead of directly using the slope of the linear curve, a new term, D, was introduced (2). The D-value of an isothermal process now possesses some biological meaning. It is defined as the time needed to achieve one log (a 10-fold) reduction in the bacterial population within the test medium under a constant heating temperature. Mathematically, D is the inverse of k:

$$\log(C) = \log(C_0) - \frac{1}{D} \times t \tag{5}$$

$$D = \frac{\ln(10)}{k} \approx \frac{2.303}{k} \tag{6}$$

Since D is directly related to the rate constant (k) under isothermal conditions, it can be used to correlate the effect of temperature on thermal inactivation of microorganisms in food systems. Historically, this correlation is also expressed as a log-linear relationship between D and T:

$$\log(D) = \log(D_0) - \frac{1}{z} \times T \tag{7}$$

In this equation, z also has some biological meaning to food scientists and food microbiologists. This term (z) is defined as the increase in temperature needed to reduce the D value by one-log cycle (a 90% reduction in D value) within the test medium. It is an indicator for the effect of temperature on thermal inactivation of microorganisms under isothermal conditions.

Determination of the kinetics data (k, $E_a$, D, and z) of microorganisms in foods is usually accomplished by graphical methods. Figures 1 and 2 illustrate the concept of using graphical methods to determine the thermal inactivation kinetics of microorganisms using the same data set and both approaches discussed in this section. These two figures clearly demonstrate that both approaches are equally suitable for kinetic analysis of thermal inactivation. However, the second approach (D and z values) are predominately used and historically accepted by food scientists and microbiologists. Therefore, the latter approach will be discussed in more detail in the following section.

## Using the Linear Kinetic Model

The D and z values provide critical information about the heat resistance of the target microorganism for a thermal process. If the food to be pasteurized/sterilized can be instantaneously heated to and held at a constant process temperature, and if the target organism is uniformly distributed within the food, then the D and z values can be directly used to accurately estimate and calculate the extent of bacterial destruction.

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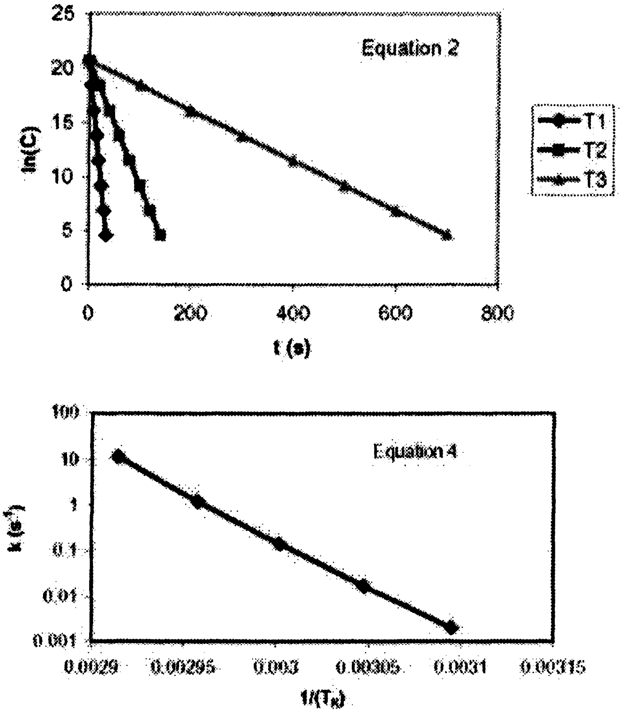

*Figure 1. Graphical representation of the $1^{st}$-order inactivation kinetics under isothermal conditions and the dependence of inactivation rate upon heating temperature.*

However, most foods cannot be instantaneously heated to a process temperature because heat transfer is a dynamic and transient process. If the food is solid, then heat must be transferred by conduction, which means that the outer layer of the food is heated first, and the center of the geometry still remains cold. This point is normally known as the "Cold Spot", or the coldest point in the food. To kill bacteria potentially present in the cold spot, heat must penetrate to this point.

To design an effective thermal process, it is necessary to identify the potential cold spot in a food, and to increase the temperature of this point sufficiently high enough to kill the microorganisms. To ensure microbial safety, it is also necessary to identify the most heat-resistant target organism of concern. If this particular organism is inactivated, then it is fairly confident to declare that the food is safe with respect to this organism.

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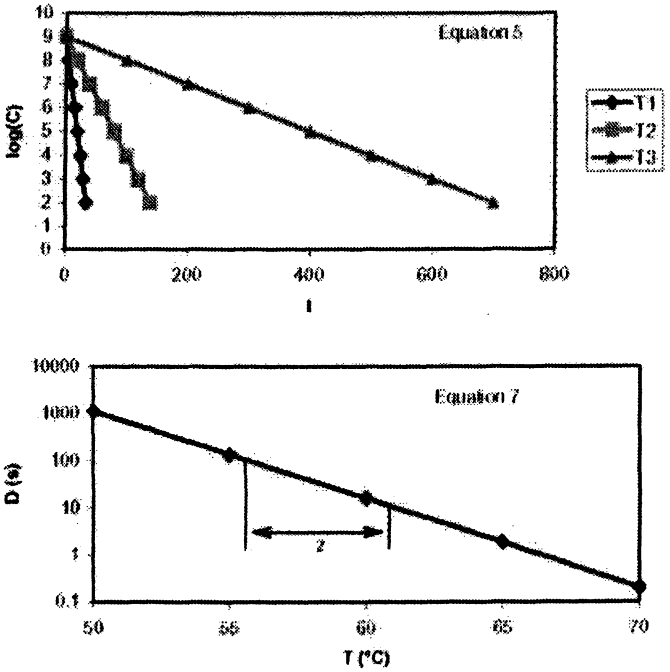

*Figure 2. Linear reduction of bacterial counts under isothermal conditions and the dependence of D values on temperatures.*

Since the temperature at the cold spot is not constant during heating, the D and z values cannot be directly used to calculate the extent of bacterial destruction. The total kill of bacteria is the integration of the entire transient process of heating. In particular, a parameter, called the F-value, which is the accumulated or integrated lethality, expressed as equivalent minutes at a specific reference temperature, $T_{Ref}$, is used for designing thermal processes (*2*). The value of F can be derived from the 1st-order kinetics (eq 1), as follows:

Separating by terms, eq 1 can be re-written as:

$$\frac{dC}{C} = -k(T)dt \tag{8}$$

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This equation can be integrated, resulting in

$$\int_{C_0}^{C} \frac{dC}{C} = -\int_0^t k(T)dt \tag{9}$$

This equation can be further written as

$$\ln\left(\frac{C}{C_0}\right) = -\int_0^t k(T)dt \tag{10}$$

By changing the base of logarithm, the destruction of bacteria can then be expressed with terms more commonly used by food scientists and food microbiologists:

$$\log\left(\frac{C}{C_0}\right) = -\int_0^t \frac{k(T)}{\ln(10)}dt = -\int_0^t \frac{dt}{D(T)} \tag{11}$$

The far left hand-side of eq 11 represents the total log reduction with respect to the initial counts of bacteria. The term D(T) in the far right side of equation represents the rate of bacterial destruction as a function of transient temperature. Since D(T) is a function of temperature (eq 7), the total log reduction of bacteria in a transient process can be expressed as:

$$\log\left(\frac{C}{C_0}\right) = -\int_0^t \frac{1}{D_0} 10^{\frac{T}{z}} dt \tag{12}$$

where $D_0$ and z are defined in eq 7. Food scientists and microbiologists compare dynamic processes with a process held under a known reference temperature, $T_{Ref}$. For this temperature $D_0 = D_{Ref}$. Thus, for any thermal process, regardless isothermal or dynamic, the estimated total log reduction during a thermal process can be normalized to a 'process equivalent' total kill under the reference temperature (eq 13):

$$\log\left(\frac{C}{C_0}\right) = -\frac{1}{D_{Ref}} \int_0^t 10^{\frac{T-T_{Ref}}{z}} dt \tag{13}$$

Since $D_{Ref}$ is the time required to achieve a 1-log reduction, the result of the right side of eq 13 is multiplied by $D_{Ref}$ to estimate $F_{T_{Ref}}$ - the total heating time required to achieve desirable log reductions at the reference temperature ($T_{Ref}$).

With the concept expressed by the F- value, the time of a thermal process can be designed as multiples (n) of the D value ($D_{Ref}$) at the reference temperature for determining the obtained lethality. If the total heating time of a thermal process is equivalent to $n \times D_{Ref}$ at the reference temperature, then the process is conventionally known as a nD process. With this concept, it is now possible to compare different time-temperature histories of any thermal process.

However, we caution that before using the above equations for integrated lethalities in designing thermal processes experiments are needed to validate these equations.

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## Nonlinear Thermal Inactivation Curves

Some experimental observations indicate that thermal inactivation curves are not always linear (*3*). One of the simplest cases where this would arise is for a culture containing two different strains of microorganisms with two distinct characteristics of thermal resistance. If each individual strain follows 1st-order kinetics, then models describing the inactivation kinetics for the mixed culture can be derived.

Let's assume that the total initial concentration of a two-strain (strain 1 and 2) mixture of organisms is $C_0$, f is the fraction of Strain 1 in the initial inoculum, and $C_1$ and $C_2$ are the concentrations of the two strains of microorganisms at any time of thermal inactivation. Since both strains follow the 1st-order inactivation kinetics, the concentrations of both strains of organisms during heating can be expressed as a function of time:

$$C_1 = fC_0 e^{-\frac{2.303}{D_1}t} \tag{14}$$

$$C_2 = (1-f)C_0 e^{-\frac{2.303}{D_2}t} \tag{15}$$

Then total log counts of microorganisms at any given time of thermal inactivation are the sum of both strains. Therefore:

$$\log(C) = \log(C_1 + C_2) = \log(C_0) + \log\left[ f e^{-\frac{2.303}{D_1}t} + (1-f) e^{-\frac{2.303}{D_2}t} \right] \quad (16)$$

Figure 3 shows the examples of hypothetical thermal inactivation curves of a two-strain mixture as affected by the relative ratio of the two strains with two different D values. As defined previously, f is the initial fraction of the strain with a smaller D value in the two-strain mixture. If f = 0, the initial inoculum only contains the stain with a higher D value. And if f = 1, then the initial inoculum consists of the first strain with a small D value. With a two-mixture inoculum, the isothermal inactivation curves show different degrees of upward concavity. Figure 3 (A and B) clearly illustrates that the strain with a larger D value should be the target organism for thermal processing when the two strains are significantly different in thermal resistance. In these curves, the D value of the first strain is only 25 or 50% of the other strain in the mixture. If both strains are in the food system at the same time, the strain with the lower D value would be preferentially inactivated. Even though the majority (90-99.9%) of the mixture is the first strain, the process is clearly determined by the inactivation of the second strain which is significantly higher in D value.

If both strains are similar in D values, as illustrated in Figure 3 (C and D), the thermal inactivation is still primarily affected by the strain with the higher D value, but the impact of the relative fraction (f) of the mixture is less significant. The curves do not show significant non-linearity, and can be modeled by 1st-order inactivation kinetics.

Figure 4 shows an extreme example of hypothetical thermal inactivation curves with dramatically different heat resistance. The D value of the less heat resistant strain (Strain 1) is only 5% of the more resistant strain (Strain 2). There are 2~4 logs of Strain 2 organism in the total population of a 9 $\log_{10}$ mixture. The inactivation curves are linear during the initial stage of heating. However, the curves all level off, showing the "tailing effect", when the more heat resistant strain is present in the mixture. In most cases, the "tailing effect" usually indicates that a small fraction of organisms cannot be inactivated within the time frame of the study. Figure 4 illustrates that the "tailing effect" may be caused by the contamination with a small fraction of very heat resistant organism. Because the contaminating strain is extremely heat resistant when compared with the target organism, they can survive even though all the less heat resistant target is eliminated from the mixture. Since the heat inactivation study is usually conducted against the target organism, the sampling time and experimental plan are developed based on its heat resistance which is very low in D value when compared with the contaminating strain. The sampling time is too small for the contaminating organism. If the same sampling scheme developed for the target organism is used to sample the contaminating organism, heating time may not be

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sufficient to cause a significant change in the population of the contaminating organism. As a result, although the heating time may seem extremely long for the target organism, the contaminating organism will survive the heating, giving a "tailing effect".

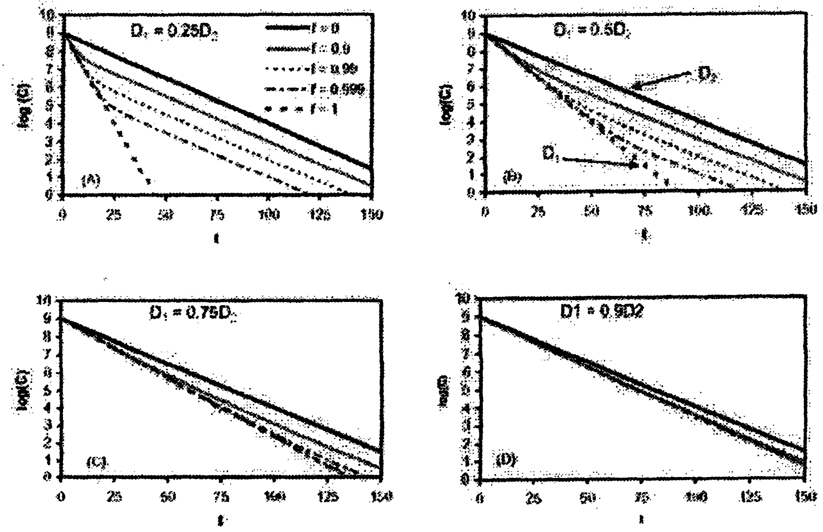

*Figure 3. Effect of mixed culture with different thermal resistance on thermal inactivation curves.*

When observing a "tailing effect", the model expressed in eq 16 can be used to show the potential existence of the more heat resistant contaminating organism. It may be necessary to conduct a differential heating study to further identify the existence of the more heat resistant strain. The differential heating study can be conducted in two phases. In the first phase of the study, heating is used to eliminate the less heat resistant strain. Then, in the second phase the remaining organism may be sub-cultured, isolated, propagated, and further studied to examine if the contaminating strain is different from the target organism. Another set of heat inactivation studies may be conducted to determine the thermal inactivation kinetics of the isolated contaminating organism.

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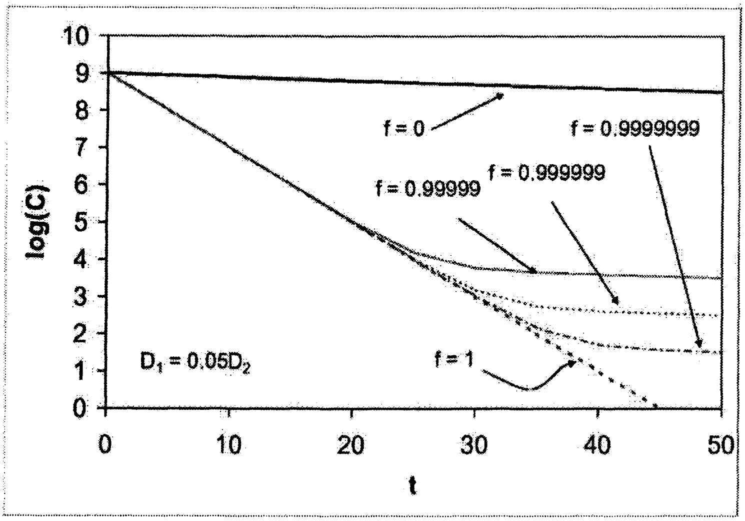

*Figure 4. Effect of mixed culture with distinctively different thermal resistance and the manifestation of "tail phase".*

## The Shoulder Effect

The shoulder effect (Figure 5) is another category of the thermal inactivation curves commonly reported in the literature. The shoulder effect is usually manifested in the initial stage of heating where the microorganisms are not inactivated by the heating. Therefore the thermal inactivation curve is "flat" during the initial stage of heating. But after passing a threshold heating time ($t_0$), the inactivation curve becomes linear, as illustrated in Figure 5.

If heat is instantaneously delivered to the food, and its temperature is immediately raised to the heating temperature, there may be some biological mechanisms responsible for the "shoulder effect" for some organisms. There may exist an initial energy barrier similar to the "activation energy" in chemistry. Sufficient thermal energy must be accumulated before showing the lethal effect. For these kinds of thermal inactivation curves, the kinetics can be modeled by:

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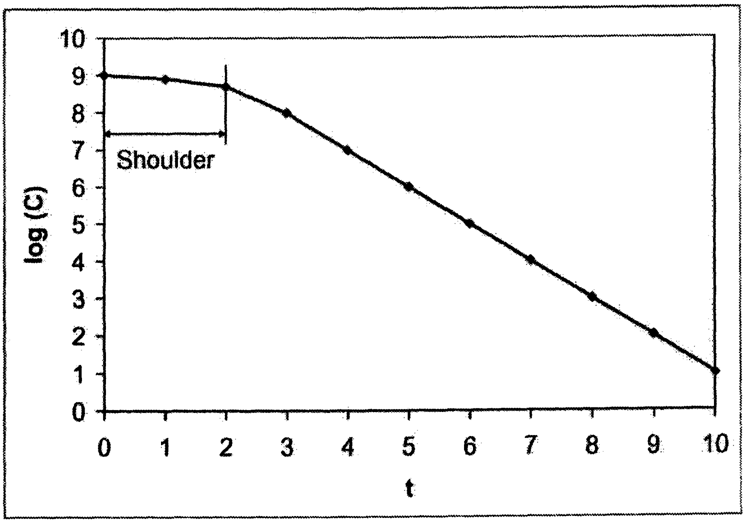

*Figure 5. A thermal inactivation curve with "shoulder effect."*

$$\begin{aligned} \log(C) &= \log(C_0) \quad at \quad t \leq t_0 \\ \log(C) &= \log(C_0) - \frac{1}{D}(t - t_0) \quad at \quad t > t_0 \end{aligned} \tag{17}$$

However, the "shoulder effect" may be caused by a simpler physical factor. Heating studies are usually conducted in some type of container. The amount of sample, thickness of containers, or existence of air pockets in the samples may affect the initial heat transfer process. The sample temperature cannot be instantaneously increased to the heating temperature used to kill the target organism. Most physical systems, such as food samples in containers, possess certain physical characteristics that affect the transfer of heat into the samples. One of the physical characteristics is the system's response time (τ), which, in this case, is defined as the time required for the temperature of the sample to reach 63.2% of the difference between the heating temperature and the initial temperature of the sample. The total time required for the food sample to reach the temperature of the heating medium is usually 3 times the response time.

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Since the kinetic data is gathered under isothermal conditions, and the temperature of the sample does not increase instantaneously to the final heating temperature, the initial stage of heating should not be included in the kinetic data analyses.

Therefore, it is necessary to measure the response time of the food sample subjected to isothermal heating. The initial sampling time must be sufficiently long ($\geq 3\tau$), particularly at the higher temperature range where the D value is relatively low. The effect of response time, however, usually does not significantly affect the measurement of D values at lower heating temperature where the D values are substantially longer than the system's response time.

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## A General Model

In the past few years, interest has increased to understand the response of a cocktail of different strains of an organism relative to thermal inactivation kinetics. In theory, the mixed culture model (eq 16) can be extended to understand the fraction and the D value of each strain in the cocktail. For organisms with similar heat resistance, the thermal inactivation curves should not deviate significantly from the linear kinetics. Therefore, the thermal inactivation curve should still show a linear trend. As illustrated in Figure 3 (C and D), the shape of thermal inactivation curves could, for practical purposes, be linear. Therefore the $1^{st}$-order kinetics (eqs 5 and 7) can be used to describe the response of the cocktail of microorganisms to heat under isothermal conditions. The D values measured under isothermal conditions would represent the overall heat resistance of the cocktail of microorganisms.

Although the $1^{st}$-order kinetics can be applied to many microorganisms, it cannot be universally applied to all categories of microorganisms. Some organisms may not follow the $1^{st}$-order kinetics and, therefore, the thermal inactivation curves may show substantial deviation from the log-linear curves. Such curves may be either convex or concave (Figure 6). Since the rate of inactivation changes continuously with time, it is not accurate to use a D value to describe the kinetics of organisms showing obvious nonlinear characteristics during thermal inactivation.

A simple generic mathematic model (*4*, *5*) has been developed to describe the nonlinear behavior of thermal inactivation. With an assumption that the rate of inactivation may change with time under a constant temperature (eq 18), the new model is capable of describing convex, concave, and linear thermal inactivation curves (eq 19):

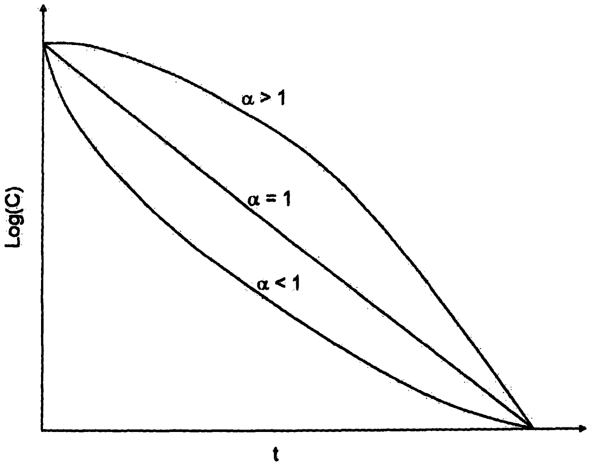

*Figure 6. A thermal inactivation may be linear, convex, or concave.*

$$\frac{dC}{dt} = -kCt^{a} \tag{18}$$

$$\log(C) = \log(C_0) + Bt^{\alpha} \tag{19}$$

In eq 19, α is a constant that determines the shape of thermal inactivation or survival curves. If $\alpha = 1$, then the equation is reduced to the linear form. If $\alpha > 1$, the semi-logarithmic inactivation curve shows downward concavity (concave). And if $\alpha < 1$, then the inactivation curve is upwardly concaved (convex), sometimes referred to as "tailing." According to Huang and Juneja (*4*), this model is more accurate in describing nonlinear survival curves. Figure 7 shows examples of hypothetical nonlinear curves shown in Figure 3 (A and B with $f = 0.999$) and described by this model. As shown in this figure, eq 19 provides a smooth and accurate description of convex-shaped thermal inactivation curves in the region shown. Figure 8 shows examples of concave curves fitted by eq 19.

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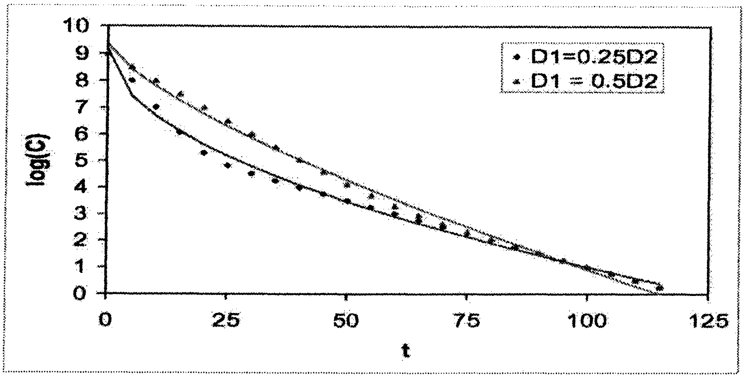

*Figure 7. Examples of convex curves (Figure 3A and 3B) fitted with the Weibull-type general model (eq 19).*

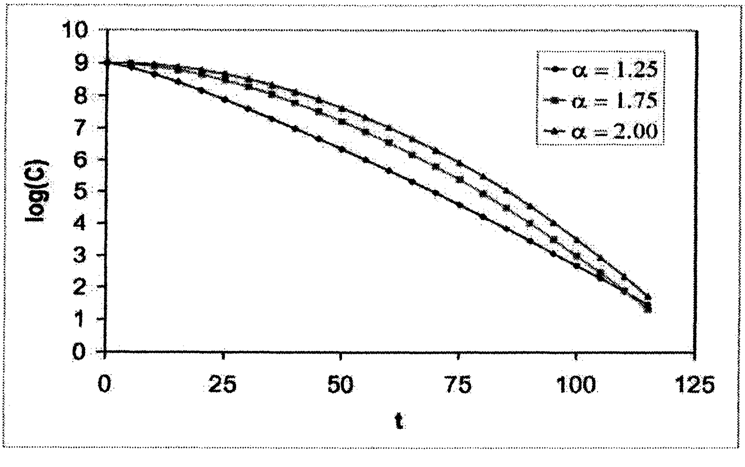

*Figure 8. Examples of concave curves fitted with the Weibull-type general model (eq 19).*

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**Table I: Some Survival Curves, ln(p(t)) versus t, where p(t) is the Probability that a Specific Cell Will be Viable at Time t. The Function h Represents the Derivative of the Survival Curve; h(0) is the Derivative at time = 0. asym. D- value Represents the Approximate D- Value for Large Times (asymptotic D- value). The Names are Those Sometimes Used to Describe the Model**

| *Model : ln(P(t)) =* | *Parameter restrictions* | *Properties* | *Model Name* |
|---|---|---|---|
| $-kt^{b+1}$ | $k > 0$, $b > -1$. | Convex ($b < 0$) or Concave ($b > 0$), no asym. D-value | Weibull |
| $-kt + \ln(1 + \frac{k}{w}(1 - e^{-wt}))$ | $k, w, > 0$. | Concave, asym D-value = $\ln(10)k^{-1}$, $h(0) = 0$ | Two stage |
| $-kt + \ln(1+bt)$ | $k, b > 0$. | Concave, asym D-value = $\ln(10)k^{-1}$, $h(0) = b-k$ | Adjusted two-stage |
| $h_0\ [(1-\alpha)t - \alpha b(\exp(-t/b) - 1)]$ | $h_0 < 0$, $0 < \alpha < 1$, $b > 0$. | Convex, asym D-value = $\ln(10)\ [h_0(1-\alpha)]^{-1}$, $h(0) = h_0$ | Modified Poisson |
| $-\alpha\ln(1+\beta t)$ | $\alpha, \beta > 0$ | Convex, asym D-value = 0 | Gamma |
| $-\ln(1+ \exp(a + b\ln(t) + ct))$ | $b, c > 0$ | Convex, asym D-value = $\ln(10)c^{-1}$ | Log-Logistic ($b > 0$) |
| $-\ln(1+ \exp(a + ct)) + \ln(1+ \exp(a))$ | $c > 0$ | Sigmoidal, asym D-value = $\ln(10)c^{-1}$ | Logistic ($b = 0$) |

References: Weibull: Huang and Juneja (*4*), Peleg and Cole (*5*); Two-stage: Juneja et al. (*7*); Modified Poisson: Hans (*8*) and Sharpe and Bektash (*9*); Gamma: Bazin and Prosser (*10*); and log-Logistic: Juneja et al. (*11*).

The above model is sometimes referred to as the Weibull model, because of its connection to a Weibull distribution of survival times (*5*). In fact, researchers over the years have developed many models, in addition to the ones described above, that describe survival curves. Examples of some curves and their properties are given in Table I. To determine the "best" model for describing survival curves, statistical analyses are needed. A good textbook to consult for modeling inactivation data is *Mixed-effects Models in S and S-Plus*, written by Pinheiro and Bates (6).

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Much research is needed for gaining an understanding of the mechanisms that lead to different types of nonlinear curves. It is possible that prior treatment - what has happened to the cells before being subjected to a thermal treatment - could significantly affect the shape of the survival curves. Even slight differences in the environment and preparation of cultures might have a significant effect on survival curves, particularly for small and large times, because such effects might affect the physiology of the cells. This effect could explain, in part, the variations seen in survival curves during experiments.

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## Chapter 17

# Computational Tools in Predictive Microbiology

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Food microbiology has been applying mathematical concepts and computational techniques at an increasing rate. One reason for this is the growing demand to analyze vast amounts of microbiology data, whose quality has greatly improved due to better and better measuring systems. Another reason is the progress in developing mathematical, computational means to process those data. The application of powerful computational tools has had a key role in the evolution of predictive food microbiology. ComBase, the No.1 database of bacterial responses to food environments introduced in this paper, is an example how the computational and mathematical tools have strengthened each other during the last two decades.

## Introduction

Hardly more than a few decades ago, food microbiology was still a descriptive science. With the advent of powerful and easily available computers, a new discipline, a quantitative approach to describe the microbial ecology of food, started to take shape. The name "predictive food microbiology" has been universally accepted for this branch of microbiology, though it could have been more aptly called "quantitative microbial ecology of food". The first book on the subject was the monograph of McMeekin et al. (*4*) that established the quantitative nature of predictive microbiology, by introducing mathematical

In Advances in Microbial Food Safety; Juneja, V., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

models to describe the effect of food environment on the microbial response. The most recent book in the field (*3*) put even more emphasis on using mathematical models. The evolution of predictive microbiology into a more exact science is well illustrated by the increasing number of food microbiology papers using mathematical modelling techniques (Figure 1).

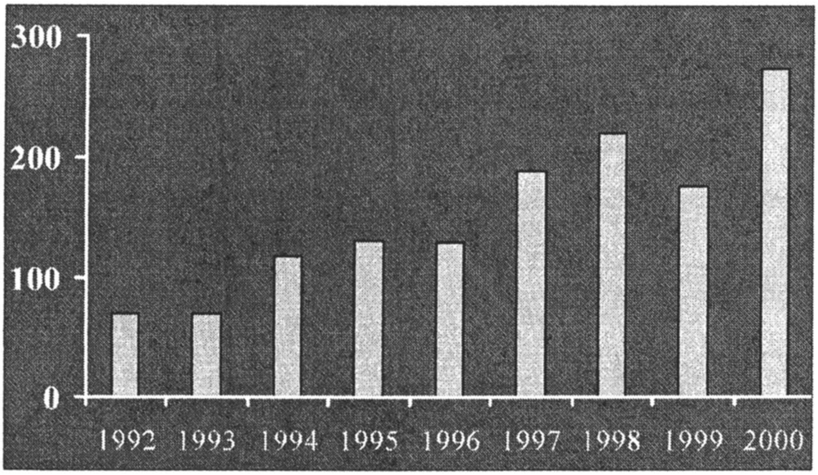

*Figure 1. Number of papers with keywords containing 'food microbiology' and 'modelling'. Source: Food Science and Technology Abstracts.*

A basic assumption of predictive microbiology is that, in a constant environment, the *relative* (or: *specific*) growth/death rate of a homogeneous microbial population is constant with time (*1*). In other words, the percentage increase/decrease of the cell population in unit time is constant. This is a simple, logical and understandable model, similar to those commonly used in physical and chemical sciences for processes such as dissipation, diffusion, etc, when the force that causes the change of a certain quantity is constant with time. The problem is that this idealistic scenario is disturbed by several intra- and extra-cellular factors. Examples for these are the physiological state of the cells, the heterogeneity of real-life microbes, the dynamically changing environment and the interactions between cells, competing populations and the environment. Still, because of the consistency of the specific growth rate of microbes in a given environment, this has remained the most important parameter to quantify the microbial response. Since the necessary direct measurements are difficult, especially at low cell concentrations, MANY data can substitute for their lack of ACCURACY. The increasing amount of data, however, needs databases and computational tools. ComBase (www.combase.cc) is such a database, a repository of measurements on microbial growth and survival in various environments. It is freely available via the Internet, and has become an invaluable source for academia, industry and regulatory officers (*2*).

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## The ComBase Story

When the UK Ministry of Agriculture Fisheries and Food initiated, in 1988, a coordinated program on growth and death of bacterial pathogens it became evident that relevant data should be collected and analyzed in a computerized and standardized way. The collected data became the basis of the first validated predictive models on the growth and survival of food-borne microbes, commercialized in a PC package called Food MicroModel. The task of supporting these developments was taken over, when established, by the UK Food Standards Agency (FSA). The FSA, in 2003, released all the data behind the Food MicroModel and funded the development of a program called Growth Predictor, by the Institute of Food Research. The program is freely available today at (www.ifr.ac.uk/Safety/GrowthPredictor). It is the result of a re-modelling effort on all the available growth data (mainly on bacterial pathogens), utilizing the scientific developments of the 1990s.

Parallel to these events in the UK, the US counterpart of Food MicroModel, called PMP (Pathogen Modelling Program: www.arserrc.gov/mfs/pahogen.htm) was developed at the Eastern Regional Research Center of the USDA Agricultural Research Service. Soon, the coordinators of these research centers and funding agencies on the two sides of the Atlantic recognized that a common, joint, database and unified model would be beneficial for everybody. This is how ComBase, the Combined Database of microbial responses to food environments (see www.combase.cc) started its life. It is now an internet-based, publicly and freely available database, for research and training/education purposes, for food microbiologists, manufacturers, risk assessors and legislative officers. The original Food MicroModel and PMP datasets have been supplemented with additional data submitted by supporting institutes, universities and companies; as well as by data compiled from the scientific literature. Under the funding of the European Union, many EU institutions are also adding their data to ComBase. As written by McMeekin (*5*), *"... ComBase can be a watershed in the evolution of predictive modelling and its widespread applications"*.

Table I summarizes the most important organisms and the respective number of ComBase records storing information on their kinetic responses to food environments. One record represents a specific combination of environmental factors to which a microbial response was recorded. The response can be either a measured viable count growth/survival curve (the majority of the records are like this) or a measured / estimated specific growth rate characteristic to the bacteria and the combination of environmental factors. Among those factors, the temperature is always mandatory to be recorded, however, the pH and water activity are not; it depends on if they were reported at all. Besides, several other factors are recorded, depending on how detailed information is available on the measurement.

The lack of compatibility between microbiological data measured by different people has always been hindering the computational, numerical processing of those data. ComBase is an example to present pooled data in a

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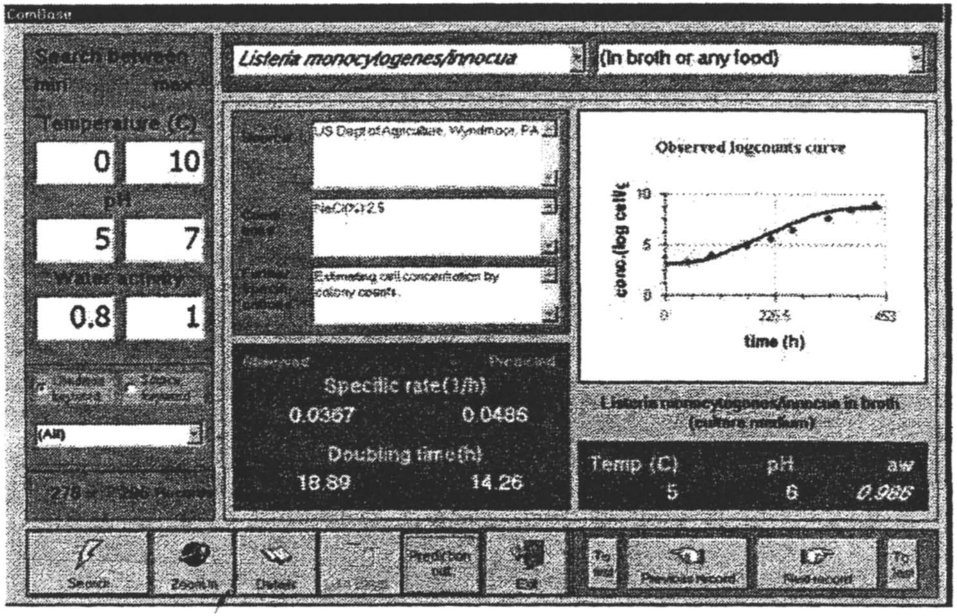
ComBase
Observed logcounts curve
conc.(log cell/
10
5
0
0
226.5
453
time (h)
Search
Zoom in
Details
Prediction out
Exit
To first
Previous record
Next record
To last

Listeria monocytogenes/innocua

(In broth or any food)

Search between
min max
Temperature (C)
0 10
pH
5 7
Water activity
0.8 1

(All)

276 of 296 Records

Source: US Dept of Agriculture, Wyndmoor, PA

Conditions: NaCl(%)=2.5

Further information: Estimating cell concentration by colony counts.

| Observed | Predicted |
|---|---|
| Specific rate(1/h) | |
| 0.0367 | 0.0486 |
| Doubling time(h) | |
| 18.89 | 14.26 |

Listeria monocytogenes/innocua in broth (culture medium)

| Temp (C) | pH | aw |
|---|---|---|
| 5 | 6 | 0.986 |

*Figure 2. A query and output screen of the stand-alone version of the ComBase-browser program. It shows that, at storage temperatures between 0 and 10°C, altogether 296 records were found on the microbial responses of Listeria, with pH between 5 and 7, and water activity between 0.8 and 1. The particular record displayed (record 276) shows a growth curve measured at 5°C, pH 6, and $a_w$= 0.986. The raw data (dots) can be compared with prediction (curve) generated by Growth Predictor.*

In Advances in Microbial Food Safety; Juneja, V., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

**Table I. Combine Records for Key Organisms.**

| *Organism* | *Number of records in Combase* |
|---|---|
| *Aeromonas hydrophila* | 2269 |
| *Aeromonas sobria* | 576 |
| *Aeromonas caviae* | 432 |
| *Bacillus cereus* | 2508 |
| *Bacillus licheniformis* | 328 |
| *Bacillus subtilis* | 914 |
| *Clostridium botulinum (prot.)* | 367 |
| *Clostridium botulinum (non-prot.)* | 358 |
| *Campylobacter* | 506 |
| *Clostridium perfringens* | 1031 |
| *Escherichia coli* | 3946 |
| *Listeria monocytogenes/innocua* | 8017 |
| *Staphylococcus aureus* | 1583 |
| *Shigella flexneri* | 745 |
| salmonellae | 4302 |
| *Yersinia enterocolitica* | 2203 |
| *Brochothrix thermosphacta* | 640 |
| lactic acid bacteria | 721 |
| pseudomonads | 504 |
| total flora | 198 |
| *Enterobacteriaceae* | 260 |
| yeast spp | 2203 |

standardized format and so to make the data available for everybody via an Internet database.

As McMeekin et al. (*6*) remarks, the Internet has been playing a similar role in the spread and availability of information as the invention of printing by Gutenberg in the 16th century. Indeed, vast amounts of information have become easily available and accessible, via the Internet, for a vast number of users. ComBase is an example for the development in "e-science".

According to John Bell, the Chief Executive Officer of the UK Food Standards Agency "ComBase is an example of the way that governments and the research community can successfully work together to help improve the safety of food products. The Food Standards Agency strongly supports this initiative, its widespread application and its use to reduce food borne disease."

Although collaboration began as an academic exercise, having a single database of information and joint models offers huge benefits to assuring the safety of foods in international trade.

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## Chapter 18

# Industry Perspectives on Performance Standards

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The food industry endorses the use of performance criteria, and where appropriate, performance standards to establish process control verification, as recommended by the National Advisory Committee on Microbiological Criteria for Foods and the International Commission on Microbiological Specifications for Foods. Performance standards are appropriate if they are justified through linkage to public health goals with measurable impact on food borne illnesses related to specific food products. When performance standards are warranted, the food industry wants all federal and state regulatory agencies to develop and apply performance standards equally for all production, processing, retail and food service sectors. The food industry believes that the development, and ultimately the impact of performance standards, will be optimized when all stakeholders participate in the process. The food industry contends that there are performance standards today that are not science-based, achievable or responsible for measurable reductions in food borne illnesses. In these instances, industry recommends that regulatory authorities eliminate these performance standards until such standards can be based and justified on principles endorsed by experts in the area of performance standards.

In Advances in Microbial Food Safety; Juneja, V., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

## Basis for Performance Standards

The food industry shares the views of the microbiological experts that comprise the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) with respect to the role of performance standards. NACMCF (*1, 2*) has supported the use of performance standards to define the expected level of control at one or more steps in a process. Industry agrees with NACMCF that microbiological performance standards are a tool to advance the microbiological safety of food products by articulating to the industry the expected level of control through such systems as Hazard Analysis and Critical Control Point (HACCP), Pre-requisite Programs, and Sanitation Standard Operating Procedures (SSOPs).

Industry agrees that one of the most important factors in establishing performance standards for foods is to be able to measure the impact of the performance standard on public health. Without specific product-handling-illness linkages, it is nearly impossible to determine whether a performance standard truly is reducing food borne disease related to a food product. For meat and poultry products, NACMCF (*1*) concluded that existing public health statistics make it very difficult to specifically attribute reductions in enteric diseases to the performance standards enforced by the Food Safety and Inspection Service (FSIS). For example, while there has been a reduction in the frequency of isolations of salmonellae from verification samples of meat and poultry products by FSIS, and overall human salmonellosis decreased 17% (95% CI = 26% to 7% decrease) between 1996 and 2003 (*3*), the proportion of salmonellosis linked to the meat and poultry supply cannot be determined from the outbreak and epidemiological data. Despite the lack of outbreak and epidemiological data, The United States Department of Agriculture's (USDA) Undersecretary for Food Safety made the claim that the reduction in salmonellosis reported by the Centers for Disease Control and Prevention (CDC) "show that USDA's science-based policies to combat deadly bacteria in meat, poultry and egg products are effective" (*4*) without reference to limitations of the CDC FoodNet data clearly stated in the CDC report (*3*). NACMCF noted that the underlying assumptions of the performance standards need to be reexamined, and recommended that before new standards are adopted for meat and poultry products, alternative approaches need to be examined; and FSIS should work in greater collaboration with the CDC to measure the impact of the performance standards on salmonellosis and other relevant enteric diseases.

Industry agrees with the conclusion of the National Academy of Sciences Committee (NAS Committee) that food safety criteria, such as performance standards that are implemented in food plants, are in many cases, not directly linked to specific public health outcomes; and thus, it is difficult to identify the

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benefits that result from a particular performance standard (*5*). The NAS Committee, NACMCF, the experts comprising the International Commission on Microbiological Specifications for Foods (ICMSF), and industry have called for improved surveillance of food borne illnesses and their root cause. They reported that the use of an independent, third party database of microbial hazards and indicator organisms might prove helpful in further reducing risks and identifying root causes of illnesses. In addition, the NAS Committee called for the use of more appropriate criteria (e.g., food safety objectives, FSOs) and analytical systems (e.g., statistical process control) to improve the government's ability to make science-based decisions relative to the development and implementation of performance standards that will have the desired public health outcomes.

The NAS Committee concluded that because it has taken a very long time to develop federal food safety regulations, and because of the myriad political, economic and social factors that affect them, some current regulations have been "left in the dust" by both science and existing processes to update antiquated regulations. Additionally, the NAS Committee reviewed the extent to which microbiological performance standards are appropriate means of ensuring the safety of selected products in a HACCP-based system, and evaluated the scientific bases for existing USDA or Food and Drug Administration (FDA) microbiological performance standards. Industry also concurs with the NAS Committee that *Salmonella* performance standards for raw ground beef likely do not reflect the overall quality of a grinding operation, but likely reflect the raw materials used in the grinding operation; that the *E. coli* O157:H7 zero tolerance standard for raw ground beef seemingly has failed to reduce the public health consequences of this pathogen on an equal cost-benefit basis; that existing and proposed stabilization requirements are not justified scientifically; and that the use of worst case scenarios is not the best approach to establishing performance standards.

Industry also agrees with the experts comprising ICMSF (*6*) who have concluded that when establishing performance criteria, including performance standards, account must be taken of the initial levels of the hazard and changes of the hazard during production, processing, distribution, storage, preparation and use. ICMSF (*6*) reported that performance criteria may be established for a wide variety of reasons, but are optimal when the risk to consumers is sufficiently high and compliance with the standard is essential for consumer protection. Industry would agree with this conclusion, but would argue that compliance with some performance standards, e.g., zero tolerance for *E. coli* O157:H7 in raw ground beef (when the food industry interprets zero tolerance as establishing an expected level of control over a manufacturing process, then zero tolerance represents a performance standard), is not achievable today, and thus, not the essential element for consumer protection (i.e., cooking). Industry agrees

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with Paoli and Fazil (*7*) who concluded that there has been limited detailed analysis of technical issues in developing and implementing risk-based performance standards for pathogens. Paoli and Fazil suggested that the standard-setting process has far more complexity than is generally accounted for in current documentation and debate.

After careful review of the bases used in establishing some performance standards by FSIS, industry must question the claim made by the FSIS Administrator in 2003 that "Our meat inspection system is based on sophisticated science." (*8*).

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## International Perspectives on Performance Standards

The food industry believes that international harmonization of performance criteria, including performance standards, and the basis on which they are set, are important scientifically as well as for trade. Internationally, performance criteria have been defined as the effect in frequency and/or concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contribute to a performance objective or a FSO. A performance objective refers to the maximum frequency and/or concentration of a hazard in a food at a specified step in the food chain before the time of consumption that provides or contributes to a FSO or acceptable level of protection (ALOP), as applicable. The FSO is defined as the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the ALOP.

The World Health Organization (WHO) Surveillance Programme for Control of Food borne Disease in Europe was launched in 1980 (*9*). The primary objectives included the identification of the causes and the epidemiology of food borne disease in Europe and collaboration with national authorities in the identification of priorities in the prevention and control of food borne diseases. Their accomplishments include the harmonization of definitions and introduction of standard codes for questionnaires to investigate outbreaks. Industry believes that this global effort should be incorporated into U.S. efforts to link food borne diseases to specific food products.

For an international perspective on the basis for performance standards, Caswell (*10*) acknowledged that the application of risk analysis principles is sought when new regulations are developed internationally. Under these principles, and in line with the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary (SPS) Measures, countries should base their regulatory actions, including the development of performance standards, on scientific risk assessment. As endorsed by industry, NACMCF and ICMSF, a country should be able to clearly link its targeted level of protection, based on a scientifically assessed risk level, to its regulatory goals and, in turn, to its standards and inspection system.

Todd (*11*) pointed out that more microbiological standards will not necessarily decrease food borne disease outbreaks associated with meat and poultry products and that routine testing will not guarantee the absence of pathogens. He pointed out that the General Agreement on Tariffs and Trade /SPS Agreement stated that SPS measures must be based on appropriate criteria, codes and guidelines developed by Codex Alimentarius Commission (Codex) and are necessary to protect public health; but he questioned whether microbiological standards are appropriate to protect public health. For example, Todd pointed out that *E. coli* O157:H7 or *Salmonella* may not be good candidates for verifying process control because they are not uniformly distributed, their numbers typically are too low for quantitative recovery, and low-cost methods for their measurement are not readily available. Todd reported that components of a scheme to manage food safety risks include objectives characterized as an ALOP or as low as reasonably achievable, risk evaluation, Quantitative Risk Assessment (QRA), FSO, performance criteria, process/product criteria, Good Hygiene Practices, HACCP, and microbiological end product criteria.

## Design of Performance Standards

Industry understands that the public expects that ready-to-eat (RTE) foods are safe to eat, and knows that consumer education on safe methods for handling and preparing raw foods cannot be overemphasized if the safety of these foods is to be assured. Although it is not always a popular issue to raise, industry must face the business realities that there will be a point at which further reductions in risks associated with specific foods may have additional costs that society is not willing to bear. For industry, there is a need to balance the benefits of risk reduction with the costs incurred. Regulatory authorities can afford to be conservative as they do not bear the direct costs of tightened performance standards, nor are they required to demonstrate that the performance standards are technically achievable.

Industry supports the experts that comprise ICMSF (*6*) and those of the NAS Committee (*5*) that recommended that regulatory authorities adopt the concept of a FSO. Industry applauds ICMSF for recognizing that since GHP and HACCP are the primary tools available to help industry control microbiological hazards in foods operations, it is essential that the technical achievability of the FSO be confirmed. A FSO, based on a tolerable level of risk, helps to establish the performance of a food process that would ensure that, at the moment of

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consumption, the level of the hazard in a food would not be greater than the FSO.

When designing microbiological standards as performance standards, the principles for the establishment of microbiological criteria developed by Codex (*12*) should be followed. These principles state that a microbiological criterion (in this comparison a performance standard) should be used only where there is a definite need. Application of the standard should be practical and technically attainable by applying GHP and HACCP. The standard should accomplish the intended purpose, e.g., reducing food borne illnesses. Industry would contend that the Codex principles developed for microbiological criteria have not been applied consistently in the design of performance standards in the U.S., particularly as they apply to meat and poultry products.

Industry agrees with the NACMCF (*1*) conclusion that microbiological performance standards should be designed to effectuate a decrease in the presence of enteric pathogens with the goal of improving public health. In the design of performance standards, the stringencies of the standards should be proportional to the risk and the public health goals; and the degrees of uncertainty must be considered when setting the stringency required of the performance standard. Industry agrees with NACMCF (*1*) that the principles for linking public health goals to performance standards via a risk analysis process, articulated by ICMSF, should be followed.

Industry supports the conclusions of NACMCF (*1*) surrounding the design of quantitative performance standards. NACMCF reported that:

- assessment of the quantitative baseline data in preparation of quantitative performance standards should identify confounding factors;
- the quantitative performance standard should be applied at the steps in the process where the samples were collected to establish the performance standard;
- the use of quantitative performance standards may be more appropriate than qualitative performance standards in achieving certain public health goals, (e.g., reducing the concentration of a pathogen may not alter the detection of that pathogen);
- quantitative performance standards may be used when verifying the ability of process steps to reduce the concentrations of pathogens of concern; and
- quantitative performance standards can be modified to reflect changes in processing technologies, the implementation of new interventions as industry best practices, and new information regarding infectious dose.

Industry agrees with the evaluation of performance standards completed by the NAS Committee (*5*) and their conclusion that improvements are needed in the design of performance standards, in particular, that FSIS needs to bring

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regulatory HACCP in line with science-based HACCP. It also agrees with these expert scientific panels (ICMSF, NACMCF, NAS) that the use of single-value, worst-case estimates as a means of considering uncertainty should be avoided, particularly when more than one factor contributes to overall public health risk. Concerning this observation, it is thought that FSIS has not likely provided the scientific rationale for their selection of microbial loads for worst-case scenarios and their use in the design of performance standards. FSIS (*13*) has stated that assumptions are "conservative but reasonable", and has stated that worst case levels are not expected to actually occur. The use of the term "reasonable" appears inconsistent with the, FSIS statement that "there is not a high degree of confidence in the magnitude of the higher levels of *E. coli* O157:H7 that might exist," and that meeting the lethality standards means that only "small numbers of reference organisms would remain viable in a worst case finished product." FSIS needs to reconcile acknowledgement of survivors in a worst case scenario for adulterants such as *E. coli* O157:H7 for which there is a zero tolerance standard.

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## *Listeria monocytogenes* Performance Standards

Since 1985, FDA has maintained a zero tolerance policy for *L. monocytogenes* in RTE foods, which are considered adulterated if *L. monocytogenes* is detected in either of two 25-gram samples. Since 1989, FSIS has maintained a similar zero tolerance policy for RTE meat or poultry products. Industry has requested that regulatory authorities consider the expert evaluation on *L. monocytogenes* completed by ICMSF (*6*) and reconsider their approach to performance standards for *L. monocytogenes* based on this expert evaluation and that of the NAS Committee (*5*). Industry contends that a substantial body of evidence now demonstrates that these zero tolerance policies are scientifically unsupportable, especially when applied to foods that do not support the growth of *L. monocytogenes*. Properly implemented, HACCP and prerequisite programs can substantially reduce the prevalence of *L. monocytogenes*. However, these cannot assure the complete elimination of the pathogen from processing facilities (*14*). The WHO concluded, "The total elimination of *L. monocytogenes* from all food is impractical and may be impossible (*15*)." NACMCF noted, "currently applied technology does not permit its eradication from the processing environment or from all finished product" (*16*). ICMSF advised, "due to its widespread prevalence in the environment, eradication of *L. monocytogenes* from the food supply is impossible" (*6*). Internationally, Canada, Denmark, the United Kingdom, Australia and New Zealand have established that zero tolerance is not an appropriate regulatory strategy for *L. monocytogenes*, and that a FSO of less than or equal to 100 *L. monocytogenes* per gram provides a higher level of protection than does a more strict tolerance of "not detected in 25 grams" (*6, 11, 17, 18*).

One of the most clearly defined positions of industry relative to a performance standard for *L. monocytogenes* is given in a petition requesting the FDA to establish a regulatory limit of 100 *L. monocytogenes* per gram in foods that do not support growth of *L. monocytogenes* (*19*). The proposal is based on evidence that consumer protection is a function of cell number, and not its mere presence. A QRA based on an extensive survey of *L. monocytogenes* in RTE foods predicted that elimination of high concentrations of *L. monocytogenes* in such foods could reduce listeriosis as much as 99.5% (*20, 21*).

There is general scientific agreement that low levels of *L. monocytogenes* are not uncommon in the food supply and that such low levels are regularly consumed without apparent harm (*6*). For example, FDA and FSIS surveillance and monitoring data before 2000 indicate that as much as 5% of some RTE foods contain *L. monocytogenes* at detectable levels (*22*). A study at retail markets in Maryland and California confirmed the presence of *L. monocytogenes* in several RTE food categories at low levels (*21*). Thus, consumers are exposed to detectable levels of *L. monocytogenes* perhaps billions of times each year (*20, 23*). The FDA/FSIS risk assessment for *L. monocytogenes* concluded "exposures to *L. monocytogenes* seldom lead to listeriosis, even among highly susceptible segments of the population"(*23*). The Food and Agriculture Organization (FAO)/WHO draft risk assessment, the ICMCF and the FDA/FSIS risk assessment have concluded that human beings often consume *L. monocytogenes* at levels of at least 100 colony forming units per gram without becoming ill (*6, 23, 24*).

The Grocery Manufacturers of America is one of the world's largest associations of food, beverage and consumer products companies. Industry agrees with its position (*25*) that regulatory authorities should limit the scope of any rules, including their performance standards, to those products that have been shown to present a bona fide risk of listeriosis. Failure to do so will only deplete already scarce food safety resources with little or no return in terms of enhanced public health.

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## *E. coli* O157:H7 Performance Standard for Raw Beef

Following an outbreak associated with *E. coli* O157:H7 in ground beef patties, FSIS implemented a zero tolerance performance standard for *E. coli* O157:H7 in raw ground beef. The concern is that this performance standard was set despite the finding that the outbreak was due to systematic, inappropriate cooking procedures at a multi-state quick service restaurant chain. Industry contends that this performance standard should be re-evaluated according to fundamental scientific considerations defined by Codex and endorsed by international experts of ICMSF, such as whether the standard is technically feasible and has a measurement in place to gauge its success. The zero tolerance policy was popular with consumers, media and legislative representatives; and thus, it apparently was deemed adequately designed and developed according to regulatory authorities.

Industry agrees with ICMSF that sampling can be used to screen out some, but not all, lots that exceed an FSO of less than one *E. coli* O157:H7 per 250 grams. Assuming random, homogeneous distribution, and testing 25 grams from each of 30 sample units, a negative result provides 95% confidence that the concentration of *E. coli* O157:H7 in the lot is no more than one cell per 250 grams. As pointed out by ICMSF, and confirmed by industry through years of testing, this sampling plan will have a difficult time detecting positive lots where the level of the hazard is not random or is much lower. Even with the above sampling plan (n=30, c=0), there is a 74% probability of accepting the lot when the proportion of positive units is 1%. FSIS should rationalize its zero tolerance policy based on this information, the heterogeneous and nonrandom nature of *E. coli* O157:H7 contamination, and the lack of statistical confidence associated with its regulatory sampling and testing program.

Industry applauds the Canadian government for its approach to *E. coli* O157:H7 in its "Policy on the Control of *E. coli* O157:H7 Contamination in Raw Beef Products" (*26*). In its guidance policy, the Canadian Food Inspection Agency recognized that zero tolerance is not the correct approach, and established a statistical confidence of 95% for detecting *E. coli* O157:H7 as acceptable.

Industry supports the effort to learn more about *E. coli* O157:H7 in the beef supply, and contends that the data gaps in this area exemplify why the existing performance standard should be re-evaluated by FSIS, and a new, science-based performance standard be established. NACMCF (*2*) concluded that baseline studies for raw ground beef components (weasand, head and cheek meat; advanced meat recovery products; low-temp rendered products, partially defatted chopped beef and lean finely textured beef; domestic trim and subprimals destined for ground beef; and imported frozen beef) are necessary to identify the contribution of these raw ground beef components to the prevalence of pathogens, and to measure indicators of process control. The effects of certain variables (e.g., geographic location, seasonality, plant size, production volume) on the prevalence and levels of particular bacterial pathogens must be taken into account.

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## *Salmonella* Performance Standards for Meat and Poultry

FSIS designed the *Salmonella* performance standards to verify the adequacy of HACCP systems, or in other words, to verify process control in slaughter and ground beef operations. The rationale for this is stated in the Pathogen Reduction (PR)/HACCP Rule (*27*). According to NACMCF (*1*), the limitations of using *Salmonella* for this purpose were discussed in the Philadelphia Report (*28*). Sperber (*29*) stated that the use of a performance standard in the context of the PR/HACCP Rule (also referred to as the Megareg) cannot be considered science-based, and reflected an inappropriate use of statistics. He characterized the *Salmonella* performance standard as perhaps "the most opaque and unfortunate flaw in the Megareg."

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Industry questioned whether FSIS had the statutory authority to take the enforcement actions laid out in the policy. On November 30, 1999, a Texas meat processor making raw ground beef filed suit challenging a suspension of inspection by FSIS for failing to meet the *Salmonella* performance standard. In a subsequent hearing on the company's request for a preliminary injunction, the judge agreed with the company and reportedly expressed concern about the scientific basis underlying the performance standard. On May 25, 2000, a decision was rendered in *Supreme Beef Processors, Inc. vs. United States Department of Agriculture* in favor of the company. A U.S. District Court ruled that FSIS lacked the statutory authority to suspend inspection due to the establishment's failure to comply with the *Salmonella* performance standard. In December 2001, the U.S. Court of Appeals for the Fifth Circuit ruled that the *Salmonella* performance standard conflicts with the statutory language in the Federal Meat Inspection Act (FMIA) and therefore is invalid. The appellate court also rejected USDA's argument that the *Salmonella* performance standard should be upheld because it serves as a measure of whether pathogens that are adulterants, such as *E. coli* O157:H7, are also present in products. The court also stated that, because the performance standard measures *Salmonella* in final product but not in incoming raw materials, it cannot "serve as a proxy for cross contamination because there is no determination of the incoming *Salmonella* baseline," a position later endorsed by the NAS Committee (*5*).

Based on FSIS *Salmonella* testing data suggesting that *Salmonella* levels in raw meat and poultry products have decreased, FSIS has proposed changing the *Salmonella* performance standards. Industry endorses the NACMCF (*1*) and NAS Committee (*5*) recommendations that the 1998-2001 FSIS HACCP verification data not be used to establish a new performance standard for ground beef or to determine either regional or seasonal variability in *Salmonella* prevalence, and that a 12-month survey, stratified by production volume, month and region, and including a sufficient number of samples, be conducted. Current verification sampling programs were not designed to provide statistically valid estimates of national prevalence and levels of microorganisms. NACMCF (*1*) also noted that, at that time, a decreased incidence in *Salmonella*, as indicated by FSIS verification sampling and testing, had not led to a decrease in disease associated with *E. coli* O157:H7 in ground beef. NACMCF stated, "In this instance, the underlying assumptions of the performance standard need to be reexamined. Before new standards or approaches are adopted, alternative standards or approaches need to be examined."

In June 2002, FSIS issued a notice (Notice 28-02) to its field inspection personnel entitled "Actions to be Taken in Establishments Subject to *Salmonella* Testing." This notice directed inspection personnel at slaughter and grinding operations to take certain actions if an establishment failed one or more sets of the *Salmonella* performance standard, and that after a third failure, the FSIS District Manager and Washington, D.C. staff would decide what, if any, actions were to be taken. The notice stated that failure "on the part of the establishment to prevent, eliminate, or reduce to an acceptable level food safety hazards, will result in enforcement actions." This language suggests that *Salmonella* in raw beef may be viewed by FSIS as a food safety hazard reasonably likely to occur, which in turn suggests the need for a critical control point for *Salmonella*. Industry contends that there was, and is today, no scientific basis for this viewpoint. The presence of enteric pathogens such as *Salmonella* in raw meat and poultry is unavoidable; moreover, the product is rendered safe by appropriate handling and cooking.

Industry believes it is important to consider the work of Sarwari et al. (*30*) when considering the impact of *Salmonella* performance standards for raw meat and poultry on public health. Sarwari et al. (*30*) stated that, if raw meat and poultry are the primary point of entry for *Salmonella* spp. into human populations, a correlation might be expected between serotypes of *Salmonella* isolated from animals at time of slaughter and those from humans. For 1990-1996, sufficient national data were available for such a comparison. Using a mathematical model to predict serotype distribution among humans on the basis of animal data, there was a significant mismatch between the serotype distributions among humans predicted by the model and those actually observed.

The food industry suggests that FSIS increase its cooperative role with industry in achieving the goals of reducing the risks of pathogens in meat and poultry, and in reducing the public health risks from these food products. One means to demonstrating this cooperation would be to credit industry with some of the success in reducing the prevalence of potential pathogens. If one were to only read the FSIS reports, one would conclude that it is only because of the regulatory enforcement that the prevalence of pathogens such as *Salmonella* is decreasing. For example, after stating that the rate of *Salmonella* in raw meat and poultry dropped by 66% over the past six years and by 16% compared to 2002, FSIS claimed that the "declining figures demonstrate that strong, science-based

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enforcement of food safety rules is driving down the rate of *Salmonella*." (*31*). Industry questions the accuracy of the phrase "science-based enforcement." This positioning reflects an apparent need by a regulatory agency to reflect an enforcement attitude against an uncooperative industry. Similarly, the agency reported that in "the past 18 months, FSIS has implemented a series of policies and directives to control *E. coli* O157:H7, *Salmonella* and *Listeria*." (*4*). Policies and directives do not "control" pathogens; only the effective implementation of control measures by industry effectuates a decrease in the prevalence of pathogens in food products. These agency-centered comments, as stated do not reinforce a successful working partnership to optimize pathogen reduction and improvement in public health.

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## Performance Standards for Broilers

Industry agrees with NACMCF (*32*) that overarching scientific considerations associated with risk assessment for purposes of developing or modifying performance standards for broilers include a current risk estimate for salmonellosis from broilers in the U.S., the potential for intervention technologies to reduce the risk of salmonellosis from broilers, a risk estimate for salmonellosis from broilers subjected to different performance standards, and the relationship of the effectiveness of control measures employed to meet a *Salmonella* performance standard to expected changes in food borne illnesses associated with other enteric pathogens. NACMCF (*32*) concluded that FSIS HACCP verification data cannot be used to establish a new performance standard for broilers as the sampling programs were not designed to provide statistically valid estimates of national prevalence and levels of *Salmonella*. New, nationwide baseline studies are necessary. When considering the effectiveness of existing performance standards for poultry products, industry agrees with the NACMCF conclusion that the link between the frequency of isolations of salmonellae on broilers from HACCP verification samples, and the estimated incidence of human cases of salmonellosis reported by CDC (*3, 33*) is not clear. NACMCF concluded that current public health statistics make it very difficult to specifically attribute reductions in enteric diseases to the performance standards; and because of this, before new standards are adopted, the underlying assumptions of the performance standards with respect to broilers need to be further examined.

The NACMCF report (*32*) also provided a list of data and research needs that industry supports. These included obtaining epidemiological data to determine the portion of salmonellosis in the U.S. population attributed to broilers, determining the extent to which cross contamination from raw broilers to RTE foods is responsible for salmonellosis, and obtaining statistically valid data for the unbiased estimation of prevalence and cell concentration levels for *Salmonella* and other enteric pathogens on broilers throughout the farm-to-table continuum. Also needed are data that relate specific process steps/interventions to changes in prevalence and cell number, characterize the impact of food handling and preparation practices as they relate to cross contamination and survival of *Salmonella*, and define the contribution of the meal components to the risk of salmonellosis. New baseline studies for *Salmonella* prevalence on broilers should be designed to capture the impact of variables such as regionality, seasonality, climate variations, line speed, and volume of production. Industry also recognizes the potential impact of other factors, defined by NACMCF, which can affect the microbiological status of live broilers, such as *Salmonella* control at hatcheries and transportation practices.

Some U.S. consumer groups have advocated establishing a performance standard for *Campylobacter* on broilers. As Denton (*34*) stated, there is considerable debate regarding the benefit to be obtained by routine testing of foods, including broilers, for the presence of *Campylobacter* and establishing a performance standard for this microorganism as part of the food safety system. Some of the complicating factors include the lack of a generally acceptable method for its isolation and recovery on a consistent basis, the lack of knowledge regarding the ecology of the organism, and the poor understanding of the environmental sources of *Campylobacter*. Industry suggests that before any performance standards are promulgated for *Campylobacter*, that FSIS use the guidance described herein to ensure standards developed are based on science and internationally-accepted principles.

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## Stabilization/Cooling Performance Standards for Meat and Poultry

Stabilization/cooling performance standards for preventing the growth of spore-forming bacteria are given in 9 CFR 318.17(a)(2), 318.23(d)(1), and 381.150(a)(2), for RTE roast beef, cooked beef and corned beef products, fully cooked, partially cooked and char-marked meat patties, and certain partially cooked and RTE poultry products, respectively. FSIS documents used as the basis for their performance standards include: *Lethality and Stabilization Performance Standards for Certain Meat and Poultry Products: Technical Paper*, FSIS, December 31, 1998, *Performance Standards for the Production of Certain Meat and Poultry Products*, FSIS Directive 7111.1, 3-3-99, and *Compliance Guidelines for Cooling Heat-Treated Meat and Poultry Products (Stabilization), Appendix B to Compliance Guidelines*, updated June 1999. The regulations and supporting documents actually specify two performance criteria for chilling, one stated and one inferred from FSIS policy (*35*): (1) there can be no multiplication of toxigenic microorganisms such as *C. botulinum*, and no more than 1-$\log_{10}$ multiplication of *C. perfringens*, within the product; and (2) *C. perfringens* shall not exceed 100,000 per gram after chilling. Industry notes that, despite no evidence that the original May 1988 guidance on chilling (FSIS Directive 7110.3) resulted in products that presented a risk to public health, FSIS tighten the chilling requirements. Even with the more restrictive requirements issued in June 1999, FSIS went on to state that there was little margin for safety with the required chilling times and temperatures. Industry is not clear on the basis of this statement by FSIS.

With the possible exception of certain heavily spiced foods that have non-inhibitory pH values, water activities, nitrite concentrations or salt levels, industry contends that the microbiological hazards, *C. perfringens* and *C. botulinum*, would be determined to not be hazards reasonably likely to occur during chilling of meat and poultry products at processing establishments. A review of the literature (*36*) indicated that meat and poultry products have been implicated in food borne illness due to *C. perfringens*. However, the actual foods implicated were foods that contain meat and poultry as an ingredient, e.g., gravy, stews, creamed turkey or chicken, meat pie, chicken salad and taco meat, and noncured meat and poultry products such as roast beef and cooked turkey (*37, 38, 39*). None of the reported outbreaks identified improper chilling of a cooked meat or poultry product in a federally inspected processing establishment.

Numerous reviews (*36, 37, 39, 40, 41, 42, 43*) have established that causes of outbreaks related to *C. perfringens* primarily are improper holding temperatures in restaurant, cafeterias, catering operations and delis. The reasons that outbreaks have not been traced to poor temperature control in federally inspected establishments include the absence or very low number of spores and vegetative cells, excellent control by establishments over chilling processes, the decline in viable vegetative cells during subsequent storage and distribution, and the inhibitory composition of the product with the inclusion of nitrite, salt and other curing agents (*44, 45, 46*).

The scientific basis, according to FSIS, for the limit of 1-$\log_{10}$ multiplication of *C. perfringens* begins with the consideration that levels of about one million cells or greater per gram are considered necessary for *C. perfringens* to cause food borne illness. When investigating outbreaks, CDC considers viable counts of *C. perfringens* of 100,000 colony forming units or greater per gram as a criterion for incriminating a food. Data from FSIS microbiological surveys led to a prediction of a "worst-case" scenario where it was proposed that there were 10,000 *C. perfringens* per gram of raw product, even though FSIS recognized that only a very small percentage had concentrations exceeding 1,000 organisms per gram; and only one sample had an estimated density of more than 10,000 cells per gram. Furthermore, FSIS baseline studies of levels of *C. perfringens* in raw meat and poultry were incomplete, as presumptive colonies on tryptose-

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sulfite-cycloserine agar plates were not confirmed, nor were vegetative cells differentiated from spores (only spores would have been of concern with respect to cooling of a cooked product).

As stated elsewhere in this chapter, microbiological experts of the NAS Committee, NACMCF and ICMSF stated that worst case scenarios should not be used in the establishment of performance standards; and industry agrees with these experts. Industry data have shown that the prevalence of *C. perfringens* in *raw* meat and poultry is very low, generally 0 to < 100/g (*47, 48, 49*). Based on their review of the literature, Taormina and Dorsa (*50*) concluded, "a spore inoculum of 100 per gram of raw meat is a reasonable target that simulates a worst case level of spores."

An extensive summary of industry data for products tested following cooling deviations shows the prevalence and concentration of *C. perfringens* in cooked products is very low, even in products that failed to meet the chilling requirements (*51*). Thus, the statistical estimates and rationale provided by FSIS (*35*) to consider *C. perfringens* a hazard reasonably likely to occur and to require a cooling performance standard for a wide range of products are not fully supported by data.

Industry contends that incorrect assumptions were made relative to the survival and growth of *C. perfringens* in the development of cooling performance standards. *C. perfringens* generally dies off during refrigeration; however, this was not factored into the determination of contamination levels likely to occur; the target temperature for control of *C. perfringens* should be 55 °F (i.e., the lower temperature limit of growth), not 40 °F; and there is no evidence that *C. perfringens* will grow at temperatures above 122 °F. Research on impact of temperature, pH, salt, salts of organic acids, and genetic factors on the growth of *C. perfringens* (*36, 52, 53, 54, 55, 56, 57*), and the survival (*55, 58*) and germination (*36*) of spores of *C. perfringens* has helped to define risk boundaries. Taormina and Dorsa (*50*) have summarized observations made by researchers and proposed that variations in experimental methods, e.g., timing of heat shocking, inoculum levels, extent of sporulation and clumping in inoculum preparations, strain selection, culture media, and prior incubation conditions, likely are responsible for the range of outcomes reported. The water activity must be above 0.93 to support the growth of *C. perfringens* and *C. botulinum* type A and proteolytic type B (> 0.965 for *C. botulinum* type E as reported in the FDA CFSAN *Food borne Pathogenic Microorganisms and Natural Toxins Handbook*). Thus, HACCP plans for products such as precooked bacon, with a water activity below 0.86, do not need to consider *C. perfringens* (or *C. botulinum*) as a hazard likely to occur (*60*).

Research, including modeling, on temperature abuse has established conditions leading to germination and growth of *C. perfringens* to unsafe levels (*53, 61*); although the limitations of modeling the germination of germination

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and growth during cooling have been summarized (*50*). The data, the initial low number of spores in cooked product, and the fact that *C. perfringens* cannot multiply below about 55 °F substantiate that noncured meat and poultry products conservatively can be safely chilled from 120 °F to 55 °F in six hours. This is the time and temperature specified in the May 1988 FSIS Directive 7110.3. The safety of commercial cooling practices has been confirmed (*48, 62, 63, 64, 65, 66, 67, 68*). Taormina et al. (*48*) stated that "even if growth in excess of one log had occurred, our survey of raw meats indicates that a minimum four-log increase would be necessary to achieve the six to seven-log colony forming units per gram necessary to cause illness," and "processed meat products cured with sodium nitrite are not at risk for the growth of *C. perfringens* during extended chilling and cold storage."

Existing scientific data indicate that the time and temperature requirements originally established for chilling roast beef (formerly 9 CFR 318.17(h)(10)(i)) can be applied to all cooked *noncured* meat and poultry products. The opinion expressed in the January 6, 1999 Federal Register notice (*69*) is sound and reads as follows:

> "Further, there is no reason why any of the cooling safe harbors for fully cooked and partially cooked products could not be used across product categories (whole, ground or comminuted), regardless of the species of origin of the tissue."

Industry would categorize their response to cooling requirements as follows. For uncured pasteurized meat and poultry products, the critical limits are those outlined in 9 CFR 318.17. For cured pasteurized meat and poultry products, outgrowth of *C. perfringens* is not a risk likely to occur, and thus, cooling time and temperature is not considered a CCP provided the product is continuously chilled from 125 °F to 50 °F within 17 hours. Cured dry sausage products are governed by 9 CFR 318.10 (treatment for destruction of trichinae, and drying at temperatures not below 50 °F), and thus, are exempt from stabilization requirements. In meat and poultry products with a water activity of less than 0.93, or acidified to a pH of less than 4.6, the growth of *C. perfringens* is controlled, thus, eliminating the need for time-temperature limits for controlling growth.

Similar conclusions to those reached for *C. perfringens* can be reached for *C. botulinum*; but the severity of botulism warrants further consideration. In contrast to *C. perfringens*, there have been outbreaks of botulism throughout the world involving both cured and noncured meats (*70*). Thus, the potential risk of *C. botulinum* growth should be considered during the hazard analysis for a HACCP plan involving cured meat production, particularly since botulism can be life threatening. However, industry contends that the no-growth performance standard during chilling for *C. botulinum* is unnecessary. There have been no incidents of botulism in the U.S. due to poor chilling of cooked perishable meat or poultry products produced under federal or state inspection, including before requirements were established (*70*). Industry data (*71*) demonstrate that the time for botulinal outgrowth at ambient temperature in freshly cooked cured meats, such as ham, would be measured in weeks in most circumstances. Industry contends that the risk of botulinal outgrowth during the chilling of cured meat and poultry products is exceptionally low and that a continuous chill from 120 °F to 50 °F in 20 hours would be sufficiently rapid to control *C. botulinum* in perishable cooked cured products.

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## Regulatory Initiatives for Performance Standards

Under the FMIA (21 U.S.C. 601 *et seq.*) and the Poultry Products Inspection Act (21 U.S.C. 451 *et seq.*), FSIS issues regulations governing the production of meat and poultry products prepared for distribution in commerce. Under the Federal Food, Drug and Cosmetic Act (21 U.S.C. 301 *et seq.*), FDA monitors nearly all other food products. The Government Accounting Office (GAO) (*70, 72*) stated that the "federal food safety system is not the product of strategic design." The disproportionate allocations of resources between the two primary agencies, FSIS and FDA, charged with ensuring the safety of the food supply are extreme. FDA has the responsibility for ensuring the safety of about 79% of the foods consumed by U.S. consumers, but does this with only about 40% of the total $1.3 billion dollar budget. This is in contrast to FSIS, where about 60% of the total budget is spent to inspect about 21% of the food supply. Furthermore, FDA-regulated products account for about 68% of the foodborne outbreaks, compared to FSIS-regulated products that account for about 26% of the outbreaks (*68, 69*). The GAO report (*72*) pointed out that the disparity also applies to the human resources in that FSIS has 9,170 employees for daily oversight of about 6,464 meat, poultry and egg product establishments; in contrast, FDA has only about 1,900 food inspectors for 57,000 food establishments. The Center for Science in the Public Interest (*73*) has examined the disparities described above and has stated a "single, independent food-safety agency – administering a unified stature – could better address the problem with food-safety inspection and regulation, including gaps in consumer protection, inadequate coordination, conflicting public-health standards, regulatory redundancies, and slow approval of new technologies." The GAO (*72*) similarly called for change, recommending an overhaul of legislation to create a uniform, consistent, and risk-based food safety policy, and a consolidation of all food safety agencies to improve the effectiveness and efficiency of the Federal food safety system. For the benefit of the entire U.S. food system, some in industry believe it would be beneficial for the U.S. to establish a unified food safety agency that would implement risk-based inspection and take one approach to the development and implementation of food safety criteria, including performance standards. There are many stakeholders that would argue that the barriers to such a paradigm shift are too great to overcome.

The PR/HACCP Rule (*27*) stated that "All slaughter and processing plants will be required to adopt the system of process controls to *prevent* food safety hazards known as HACCP." Industry questions FSIS' conclusion that HACCP plans can "prevent" all hazards. HACCP plans typically help to reduce and control hazards, and in fewer instances, where there are kill steps, to eliminate hazards. Under the regulations in 9 CFR 417, critical limits must be designed to satisfy relevant FSIS regulations, including performance standards. FSIS stated that developing HACCP systems around verifiable, objective performance standards is the most effective way for establishments to consistently produce safe, unadulterated meat and poultry products. FSIS (*27*) stated that they wanted to *minimize* regulatory burdens on the industry, and that the performance criteria would be implemented on the basis of a statistical evaluation of the prevalence of bacteria in each establishment's products measured against the nationwide prevalence of the bacteria in the same products. Industry contends that FSIS places maximum regulatory burdens on industry through HACCP and performance standard regulatory enforcement actions, not the *minimal* regulatory burdens suggested in their policy.

When industry reviews the regulatory approach to performance standards, a major disappointment is the lack of involvement of all stakeholders in the process before performance standards are published. Industry acknowledges the post-publication comment period, but contends that better performance standards could be developed if the process was transparent and open to all stakeholders earlier, with full public disclosure and debate. Through such a process, all data and data gaps, social and political concerns, risk assessment and risk management issues, and risk:benefit analyses could be debated. The compromises would be gained through consensus-building such that the final performance standard or criteria would have a greater likelihood of being embraced, or at least accepted, by all stakeholders at the time of publication. Sperber (*29*) has characterized the government approach to science-based HACCP regulations and performance standards as "opaque legislative based systems," in contrast to "transparent science-based systems."

FSIS (*74*) stated that the use of microbiological performance standards is part of a fundamental shift in FSIS regulatory philosophy and strategy, from command and control (telling how) to performance standards (express the objectives without specifying the means). Industry contends that as much as FSIS has spoken about changes to the inspection system, there is a lack of evidence that they have moved away from command-and-control inspection

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where the use of noncompliance records, 30-day letters and notices of intended enforcement are issued as powerful regulatory instruments. Industry claims that the majority of unionized inspection staff, by-and-large the same inspection staff that was in existence before the HACCP Rule, still operates under command and control, rather than a cooperative, educational process with the establishments producing meat and poultry products. In the 1996 PR/HACCP Rule, FSIS stated that they were working with industry, academia and other governmental agencies to *develop and foster* measures that can be taken on the farm and through distribution and marketing of animals to reduce food safety hazards associated with animals presented for slaughter. Industry has yet to see the measures on the farm and throughout distribution and marketing of animals. There has been a lack of validated on the farm, distribution and marketing measures *developed and fostered* by FSIS as proposed in 1996. FSIS (*75*) stated that the PR/HACCP regulations "provide enormous flexibility for the industry to develop and implement innovative measures for producing safe foods." Industry has not yet seen the measures of progress on this initiative. Only in 2003 did they create an Office of New Technology, and there have been examples of relatively straightforward interventions (e.g., higher levels of organic acids, hydronium ion formulations, chlorine dioxide, carcass irradiation) taking months, if not years to move through the approval system that was supposed to "remove unnecessary obstacles." Besides their claim that they would remove unnecessary obstacles to innovation, FSIS (*27*) stated that they would reorganize to implement a modernized system of inspection and begin a public process to develop and evaluate new approaches to inspection, anticipating a major redeployment of its inspection resources to successfully implement HACCP and better target food safety hazards during transportation, storage and retail sale. Industry has not seen any significant redeployment; in fact, the new FSIS positions have focused on production processes, rather than transportation, storage and retail sale.

Because the regulatory system lacked any performance standard for harmful bacteria on raw products (other than *E. coli* O157:H7 on raw ground beef), FSIS (*27*) stated that its inspectors had no adequate basis for judging whether establishments producing raw meat and poultry products were dealing effectively with microbial food safety hazards. The proposed targets for *Salmonella* were intended as an initial step toward defining the levels of food safety performance that establishments would be required to achieve consistently over time. This was the first step toward the eventual incorporation of microbial testing as an integral part of process control validation and verification in facilities operating under HACCP. The FSIS approach to performance standards has been to first define a *worst-case* raw product; NACMCF, the NAS Committee and the international experts of the ICMSF do not agree with this approach to establishing performance standards. For example, consider the worst-case scenario used by FSIS to establish its lethality performance standards. For ground poultry, FSIS computed that one could be 99% confident that as many as 1.3% of samples could contain 2,300 salmonellae per gram; but the worst case scenario is based on the 97.5% upper confidence bound for the 2,300 measure, or 37,500 organisms per gram, assuming a 30% recovery rate. Assuming a serving size of 143 grams of product (pre-cooking mass), this computed to an initial load of nearly 10 million organisms per gram. Meeting the performance standard for ground poultry, there would be a 0.0174% probability that more than four organisms would survive, or an expected once in every 5,750 times. Industry contends that the use of such worst-case scenarios to establish performance standards demonstrates that recommendations made by ICMSF, the NAS Committee and NACMCF have not been fully considerable. Another example of FSIS creating performance standards that were not developed under principles endorsed by the experts of ICMSF and NACMCF were those related to the HACCP-based Inspection Models Project (*76*). FSIS stated that, while no system is perfect, the models project was an effort to reduce and eliminate defects that pass through traditional inspection. Under the models project, performance standards were based on improving what was achieved under the existing traditional method of inspection. It seems that instead of being a science-based justification for the performance standards, plants entering the project must improve their process in order to meet the new, arbitrary performance standards.

FSIS (*77*) has published guidance (FSIS Directive 5000.1) for their inspection staff on "how they are to protect the public health by properly verifying an establishment's compliance with the pathogen reduction, sanitation, and HACCP regulations." These procedures are prescriptive and involve verifying sanitation performance standards in 9 CFR 416.2-416.5 involving grounds and pest control, construction, lighting, ventilation, plumbing and sewage, sewage disposal, water supply and water, ice, and solution reuse requirements, dressing rooms and lavatories, equipment and utensils, sanitary operations, and employee hygiene. FSIS has not clearly defined the science behind these regulations to clarify when specific violations can lead to a noncompliance record stating that a meat or poultry product is adulterated and has "been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health." Inspection personnel are only required to be of the opinion that conditions *may have* caused product to be contaminated with filth or cause product to be unsafe. FSIS gives their inspectors the right to use professional knowledge and judgment in making the determination whether the sanitation performance standard requirements are met. Industry contends that there is no visible or transparent process in place to measure the abilities of inspection staff to correctly make such judgments.

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While establishing fewer performance standards than FSIS, FDA also has developed performance standards that some believe need work (*29*). The FDA HACCP regulations and performance standards on juice production do not require a pasteurization step, exempt retail establishments that produce juice for direct sale from the regulations, allow a *cumulative* five-log reduction (in contrast to a more traditional CCP philosophy), and allow product testing when complete process control measures are not applied. Sperber (*29*) contended that as "little as 20 ml of juice from one week's production (up to 1,000 gallons of juice) needs to be found free of *Escherichia coli* to provide assurance that the entire week's production is safe for consumption."

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## Microbiological Testing and Validation of Control Measures

Industry agrees with ICMSF (*6*) that food safety management systems based on preventing hazards through GHP and HACCP are much more effective in ensuring safe foods than is end-product testing. In fact, these international experts expressed concern over the "continued indiscriminate use of microbiological testing of the end product." ICMSF concluded that microbiological testing can be useful in management of food safety, but tests should be selected and applied with the knowledge of their limitations. Regulatory agencies have begun to use the wording "statistical based sampling and testing" in their policies and directives. However, these same agencies are not fully delineating the statistical limitations of sampling and testing plans, nor linking the limitations to the performance standards, particularly the zero tolerance performance standards. Testing should be done at those points where information about the microbiological status of a food will prove useful for control purposes. ICMSF (*6*) experts reported two uses for microbiological criteria: validation that control measures lead to compliance with the performance criteria and determination of the acceptability of a food when no other means of such determination is possible. ICMSF concluded that while microbiological criteria have played an important role in defining what has been acceptable, their use in testing of food has seldom proven to be effective for control of microbial hazards. Whether performance standards call for zero tolerance or establish an acceptable tolerance for pathogen prevalence, they translate into the requirement for microbiological testing by industry to verify compliance. Often it appears that testing, whether by government or by industry, is seen as the means to prevent microbiological hazards from reaching the consumer. Industry does not fully support this approach to the control of microbiological hazards.

Industry is required to establish control measures that result in processes and products that meet performance standards established by regulatory authorities, regardless of whether the performance standards are achievable with existing technologies (e.g., zero tolerance for *E. coli* O157:H7 in raw ground beef components). This creates situations where the science clearly establishes the inability to be in compliance, yet regulatory HACCP demands "artificial compliance," that is, where control measures reduce levels as low as possible, or below detectable levels, even though clearly a zero tolerance in not achievable. Thus, the challenge becomes one of validating that a control measure achieves an unattainable goal. Clearly, this is not an approach that any scientist wishes to undertake; but often the regulatory approach to HACCP leaves industry with no other option. Other complicating factors surrounding validation include the variation in acceptance by local regulatory authorities of published literature as satisfactory validating documentation and the lack of sufficient scientific knowledge and training by those in decision-making positions within the regulatory field operations staff. A regulatory authority needs only to question the legitimacy of the validation documentation, without providing a rationale for its questioning, or without providing an expectation for what is required to address its question. That is, the establishment can be left guessing as to what is required to satisfy a local authority, and have no guarantee that the validation data, even if scientifically sound, will prevail in satisfying a regulatory authority. As a result, acceptance of validation data is somewhat arbitrary, as regulatory authorities have not established, in most cases, specific criteria for acceptable validation data. Industry contends that until such criteria are established, or a set of validation documents is recognized for specific CCPs, the ambiguities and inconsistencies will persist as challenges for industry and regulatory agencies.

FSIS (*78*) states that the inspection authority should use professional judgment on how much supporting documentation to request, and not just arbitrarily ask for supporting documents. FSIS has implemented additional training to enable its inspection staff to assess the scientific and technical information that an establishment might have to support its HACCP system. Whiting (*79*), of FDA, has recognized that performance standards have led to the need for validation studies that involve decisions about the use of pathogens or surrogates, using laboratory or in-plant data, and acceptance of validation studies by the regulatory authority. Industry believes that because of these limitations in field staff expertise, and these challenges in regard to scientific validation, regulatory authorities should focus on a cooperative, mutual knowledge-building effort with industry, rather than taking enforcement positions with industry on validation data.

The regulatory agencies have significant opportunities to improve their approach to the use of performance standards. The agencies can take advantage of the expertise of international experts of ICMSF, and include, the recommendations of the NAS Committee and NACMCF to help make the process of establishing performance standards more science-based and

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transparent to all stakeholders. The agencies can take advantage of enormous financial resources provided by U.S. citizens to redefine a more cooperative approach with industry to establish and achieve mutual food safety goals and objectives. The federal government can acknowledge the limitations of the current multi-agency food safety organization and recommend changes, consolidation and cooperation that will deploy the financial and human resources against the prioritized risks facing the food industry, without regard to preconceived boundaries. The regulatory agencies governing the food industry need leadership from legislative and administrative offices to make the paradigm shifts.

Industry must continue its effort to continuously improve their operations to further reduce risks associated with food production. They must use their scientific expertise, and that developed through cooperative relationships with academia and government researchers, to seek new solutions to food safety risks. Industry must recognize that governmental regulatory agencies will need constant attention to affect their rule-making processes and outcomes. Industry must operate in an environment of legislative and regulatory HACCP, and their associated performance standards, until the agencies tackle the challenging recommendations presented above. The direction for improving the use of performance standards will be focused when the food borne disease surveillance system defines a clearer link between food products and food borne illness. Prevalence and epidemiological data, collected through statistically designed surveys, will help facilitate the prioritization of food safety risks across the entire food supply chain from production to consumption. Establishing measurement systems that allow government and industry to determine progress against food safety goals, without a pre-designed negative consequence for industry working toward continuous improvement, will lower food safety risks. There has never been a more opportune time to leverage the expertise in industry, academia and government to transform the U.S. food safety management system into a world-class program; but it will require that government regulatory agencies demonstrate courage and transparent leadership.

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## Chapter 19

# Regulatory Perspective on Enhancing the Safety of Foods Needs and Challenges

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Most food safety hazards are invisible to the eye; however, federal food safety laws, which date back a century, emphasize removing recognizably unwholesome foods from the marketplace. These laws prescribe how, where, and when food inspections are to be performed. Current understanding of the natural history of foodborne pathogens supports a more integrated (farm to table) food safety strategy. In the 1990s, Hazard Analysis Critical Control Point (HACCP) regulations were developed to delineate the appropriate food safety responsibilities of industry and government. In the 21st century food safety agencies will need to continually invest in scientific infrastructure and maintain an expert workforce to prioritize and manage evolving foodborne hazards. Appropriate levels of funding and recruitment are essential to accomplish these goals.

U.S. government work. Published 2006 American Chemical Society

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ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

## 1. Historical Context

USDA's Food Safety and Inspection Service (FSIS) has regulatory responsibility for the safety of meat, poultry and egg products and the Food and Drug Administration (FDA) is responsible for assuring the safety of most other foods. The enabling laws for both agencies were written in 1906, the year in which Upton Sinclair published "The Jungle," (*1*) a novel decrying sanitary conditions in turn-of-the-century slaughter plants. The 1906 Food and Drug Act (*2*) and Federal Meat Inspection Act (*3*) reflected a social context of the early 1900s that associated "adulteration" of food with human disease (*4*) The statutes contain parallel language prohibiting "adulteration" of food with a poisonous or deleterious substance which may render it injurious to health or any filthy, putrid, or decomposed substance that is unsound, unhealthful, unwholesome or otherwise unfit for human food. Although both laws were substantially revised in 1938 and 1967, the term "adulterated" remains the basis for both FSIS' and FDA's authorities, and does not fully reflect the natural history of most foodborne infectious diseases.

Many foodborne hazards of the early 21st century are caused by microscopic pathogens (e.g., *Salmonella, Campylobacter*) that have no effect on the appearance of food or health of animals and plants (*5*) FSIS has classified only a handful of pathogens as adulterants, which are not permitted in foods (e.g., *E. coli* 0157 in ground beef, *Listeria* and *Salmonella* in ready to eat foods, BSE infected cattle tissue). The brevity of the list indicates the resistance to declaring pathogens as adulterants.

## The FDA System

Under the FDA system, companies are legally responsible for producing foods that are not adulterated. In 2000, the agency had a workforce of approximately 250 food inspectors who were responsible for inspecting tens of thousands of food operations. Since it is not possible to inspect all plants on even an annual basis, instead FDA uses regulations and guidance to inform food companies of their requirements to produce foods that are not adulterated. FDA conducts discretionary inspections based largely upon need. Regulatory options to remove adulterated foods from the marketplace include voluntary recall by the food manufacturer. Like FSIS, in cases of noncompliance with an FDA requested recall, the FDA can initiate a court action to seize a food product or direct the producer to stop manufacturing it (*5*, *6*).

This approach works well almost all of the time since the vast majority of food producers take their food safety responsibilities very seriously. On rare occasions, problems occur when companies fail to meet their obligations or

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when emerging food safety hazards are first recognized. One example was the delayed response to *Salmonella* Enteritidis contamination in eggs. Applied research was needed to determine how to detect contaminated flocks and what interventions would prevent human illnesses. Only after agency responsibilities were clearly established did FDA begin to test flocks implicated in human outbreaks. This action provided an incentive for egg producers to implement controls and was followed by a decline in human *Salmonella* Enteritidis infection rates (*7*).

## The FSIS System

The FSIS system requires a critical inspection of every animal carcass that is destined for human consumption by touch, feel, and smell; a process referred to as organoleptic inspection. Daily inspection is also required in food operations that manufacture food products containing meat. To meet these requirements, FSIS employs approximately 7600 inspectors in 6500 plants across the nation. Plants that produce adulterated meat or poultry products can have the FSIS mark of inspection withheld, precluding interstate commerce (*5*).

As the role of pathogenic microorganisms in foodborne infections was recognized over the past century, it became clear that there was a need to shift from organoleptic to science-based inspection to address foodborne hazards of meat and poultry. Specifically, the leading foodborne pathogens in meat and poultry (i.e. *Campylobacter, E coli* O157:H7, and *Salmonella*) often colonize the intestines of healthy animals and are not detectable by visual inspection (*4*). In the worst cases, the USDA mark of inspection may have provided a crutch for plants that chose to avoid responsibility for their sanitation and food safety programs (*5*) After FSIS was criticized for not declaring *E. coli* O157:H7 to be an adulterant of ground beef, HACCP-based regulations were developed, signaling a new emphasis on industry food safety responsibilities.

The evolving view of meat inspection emphasizes preventing distribution of meat and poultry containing high loads of pathogens capable of affecting humans. Ultimately, inspection could provide an opportunity to inform suppliers and producers of hazards and to encourage best food safety practices prior to slaughter and processing, (*8*) including on-farm HACCP (epidemiological) programs to manage risk (*9*).

## Fragmented Federal Authority

A 1993 report (*10*) estimated that at least 12 federal agencies enforce 35 statutes involving food safety. These include agencies with oversight of school

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lunch programs, seafood inspection, and International Passenger Vessel Sanitation. The fragmentation of responsibility has contributed to delays in the past like the emergence of *Salmonella* Enteritidis contaminated eggs, mentioned above. After CDC reported a threefold increase in human infections (*11*) associated with internal contamination of eggs (*12*) in 1988, a decade passed before a decline in human infections became evident (*13*). Initially USDA's Animal and Plant Health Inspection Service addressed the problem as an animal disease. By the mid 1990s, FDA took the lead in testing egg laying flocks that were implicated in outbreaks of *Salmonella* Enteritidis infection. There are many similar examples of close jurisdictional boundaries. For example, USDA inspects pepperoni pizza and open faced meat sandwiches while FDA inspects cheese pizza and meat sandwiches with two pieces of bread.

There have been proposals to consolidate food safety programs into one agency to improve use of resources, consistency of strategies, and accountability; (*14*) however; pressure has not existed for such complete restructuring. Many officials and advocates recommend change within context of the laws of existing food safety agencies. This is the context in which HACCP regulations evolved.

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## International, State and Local Agencies

Local agencies, foreign governments, and international agencies have major roles in food safety policy. The economic clout of foreign nations was evident when the United States major trading partners banned the importation of beef from the United States in December 2003 after a case of BSE was reported in Washington State. The market for more than one tenth of United States beef immediately disappeared. International agencies (e.g., World Trade Organization) seek to harmonize food safety standards so that foods meet equivalency requirements (*6*) and avoid trade barriers (*15*).

Federal agencies work with state and local officials to formulate the Federal Model Food Code (*16*), which serves as a guide for state and local law. State and local agencies also participate in cooperative programs to assure that eggs, milk, shellfish, and other food commodities meet minimum food safety standards. Typically, it is county and municipal authorities that license groceries and restaurants or close retailers because of sanitation problems or outbreaks.

## Epidemiology and Regulation

The Centers for Disease Control and Prevention (CDC) is the federal agency with lead responsibility for foodborne disease surveillance. FDA and FSIS use these data to develop strategies and evaluate the effectiveness of programs. The interest in epidemiological data is evident in FDA and FSIS collaborations with CDC and states on surveillance programs such as FoodNet (*17*) and the National Antimicrobial Resistance Monitoring System (NARMS) (*18*) and laboratory subtyping (PulseNet) (*19*).

CDC also assists with outbreak investigations at the invitation of states. Often, food safety agencies conduct parallel studies to understand factors that contribute to outbreaks (*20*, *21*). As other examples of circular consultations, CDC epidemiologists consult with regulatory officials to gain in-depth knowledge of specific food industries and FDA and FSIS rely on findings from outbreak investigations to guide sampling and regulatory actions (e.g., recalls).

Epidemiology has steadily evolved with society since the mid 1800s, when John Snow investigated a cholera epidemic in London (*22*). Thus, in the 1950s, the church ice cream social was a typical foodborne outbreak scenario. It was often possible to infer the source of illness because people knew each other and had eaten together. With the advent of mass food distribution, a new outbreak scenario began to occur, in which patients did not know each other and were geographically dispersed. For example, in 1994 an outbreak of salmonellosis was associated with ice cream that became cross-contaminated when ingredients were hauled in tankers that had carried unpasteurized eggs. The ice cream was delivered to homes throughout rural America (*23*). Critical elements for investigating this outbreak included laboratory subtyping to discriminate outbreak-associated-infections from background-infections and interviews with cases and controls (healthy people) to identify the source.

Regulatory agencies have become increasingly at ease with epidemiology as a basis for enforcement action. In the early 1990s, FDA required microbiological confirmation before acting on epidemiological evidence; however, after a 1996 outbreak of cyclosporiasis (*24*), FDA banned import of implicated Guatemalan raspberries based solely on a statistical association between exposure and illness. The pathogen was never found in berries because it was present at low levels, unevenly distributed, did not grow on standard media, and was not detectable — even with sensitive testing methods (e.g., polymerase chain reaction).

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## 2. New Approaches for New Problems

### The 1993 Outbreak of *E. coli* O157:H7 in Seven Western States

A major event in the United States food safety system was the 1993 outbreak of *E. coli* O157:H7 infection (*25*) linked to a fast-food hamburger chain. With approximately 500 confirmed infections, 41 cases of hemolytic uremic syndrome (mainly in young children), and at least four deaths, (*26*) the outbreak strengthened calls for meat and poultry safety reforms. The existing balance of responsibilities for food safety among consumers, industry, and government was immediately called into question (*5*). Industry had maintained that raw products were not intended to be pathogen free and it was the responsibility of consumers to cook them so that they did not cause illness (*4*). Contamination of ground beef with *E coli* O157 presented a new and unacceptable health hazard. This low dose pathogen (*21*) caused severe illnesses and deaths in children who ate hamburgers that were pink at the center, an accepted behavior reported by 29% of U.S. adults in 1993 (*27*). The outbreak triggered demand for safe ground beef, regardless of cooking practices. The shift in responsibility to industry and government was a major impetus for FSIS and FDA to develop Hazard Analysis Critical Control Point (HACCP) regulations. The outbreak heightened awareness of many food safety challenges and opportunities. A decade later it continues to influence the food safety agenda pertaining to animal production, risk assessment, consumer outreach, market-based incentives, research and infrastructure.

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## HACCP

Hazard Analysis Critical Control Point (HACCP) programs for meat, poultry (*28*) and seafood (*29*) emphasize the distinct responsibilities of manufacturers and government for food safety. The intent of HACCP is to delineate these responsibilities based on appropriate roles. Companies are responsible for producing safe foods and government provides oversight of these processes. This approach is a departure from the historical food safety relationship of industry and government, in which companies produced the food and food safety agencies inspected them for safety. Limitations of the previous approach were made evident by limited resources within FDA and FSIS and delayed action on emerging foodborne pathogens.

Under HACCP, companies determine which hazards are likely to occur in their products and where those hazards are introduced. Controls are developed to address these hazards, and validated monitoring schemes are used to ensure that the system works. Government officials review HACCP plans and verify performance standards to assure that companies meet minimum food safety expectations (*4*). Under HACCP, FSIS shifted its role from prior-approval of blueprints, sanitation and equipment. HACCP rules require industry to provide data documenting that their equipment, facility, and food safety system is appropriate and adequate. Managers decide how a plant operates. Federal inspectors verify that the plans are appropriate, that written procedures are followed, and that the company meets minimum performance standards.

Most producers have accepted HACCP. Only a few plants have failed to develop HACCP plans that define hazards and control points. On the other hand, some producers have resisted full use of HACCP as an iterative tool to improve food safety process on a continual basis. An assessment of the reasons (or excuses) offered by industry for resistance to HACCP identified three basic categories: fear of repercussions, calls for more science, and cost (*30*). Since 2001, both FDA and FSIS launched mid-course review/in-depth HACCP verification efforts (of seafood and beef grinding establishments, respectively) to bolster compliance with HACCP regulations.

Regulatory agencies need to anticipate change (e.g scientific advances and new public policies). As industry performance improves, new pathogens emerge, new technology is developed, and court decisions demand; it may be necessary to revise performance standards that were established to assure that establishments are in complianc with requirements of HACCP regulations.

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## Animal Production

The National Academy of Science has recommended on-farm programs to control foodborne hazards (*31*); however, FSIS does not have regulatory authority over animals before they arrive at slaughter plants. Cooperative approaches are therefore needed to address the microbial quality of feed and water, sanitation, and pest control (*8*). The advent of animal identification technology (*32*) may allow animals with optimal food safety risk profiles to be processed in advance of other animals (*33*). This approach would introduce new incentives for best production practices. For example, restrictions could be placed on the use of carcasses with high pathogen loads (e.g., use for cooking). In addition, verification sampling at the processing level would provide data to suppliers and producers for food safety assurance and/or improvement.

## Risk Assessment

Risk assessments are required by the Office of Management and Budget for economically significant regulatory decisions to assure that policies are sound and cost-effective. Legislation passed in 1994 that created the Office of Risk Assessment and Cost Benefit Analysis, charged with reviewing food safety regulations to assure that they are based on sound analysis of risks, costs and benefits. Epidemiological and experimental data are used in risk assessments in the development of new policies. Epidemiological data is valued in risk assessments because it reflects experience (e.g., associations from case-control studies), provides data on trends and the burden of illness, (*34)* infectious dose, (*21*) and attributable risk (*35*). Experimental data provide additional insight into issues including the ecology of a pathogen, virulence, strain diversity, and host specificity.

## Consumer Outreach

Consumer surveys are used to develop consumer outreach on foodborne disease prevention (e.g., recommendations to clean, cook, separate, and chill) and to measure the effectiveness of interventions (*36*). Surveys are also used to identify groups with high rates of risky behavior (2*4*) and estimate the proportion of the population that is predisposed to infection by underlying disease (*37*). The best surveys use well designed questionnaires, sampling, and analytic methods.

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## Market Based Incentives

The marketplace provides economic incentives for food producers to conduct food safety monitoring (*38*). By demanding product testing and process controls, large fast food restaurant chains have created a market in which the microbial safety of ground beef is paramount. International trade has also stimulated food safety markets. Foreign buyers who demand high safety standards and pay premiums for guaranteed contracts have fueled demand for ground beef with the highest safety assurance. By securing these reliable contracts, meat processors realize benefits for their investments in food safety technology. Government can raise industry food safety standards through policies that build on these market incentives. Indeed, the combination of market and regulatory forces may have contributed to a recent decline in human infections caused by *E coli* O157:H7 in the United States (*39*).

## Research and Infrastructure

Food safety agencies must invest in their own scientific infrastructure to signal commitment to their mission and encourage innovation by industry. The simultaneous revolutions in information technology and molecular biology illustrate the need for continuous workforce development. Food safety agencies need an expert workforce for 21st century inspection, HACCP verification, to subtype pathogenic bacteria, and support epidemiological/environmental investigations. A skilled multidisciplinary team is also necessary for risk assessment model development, consumer outreach, and implementation of new food safety technologies for production and processing.

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Food safety policies have enormous influence on research priorities within the scientific community. By communicating policy and research needs, agencies generate interest and encourage funding for applied research. As an example, after FSIS classified *E coli* O157:H7 to be an adulterant of ground beef in October 1994, the number of PubMed "hits" for the search term "*E coli* O157" dramatically increased (from 45 in 1994 to greater than 300 per year from 1999 through the present). Research progressed on topics ranging from *E coli* O157:H7 ecology (*40*) to interventions for cattle (*41*). In addition, the time to obtain *E coli* O157 test results decreased from 3-5 days to 1-2 days (*42*).

## Priorities

New food safety hazards are continually being identified, increasing expectations for regulatory agency action. In some instances foods have both beneficial and adverse effects (e.g., fish can be contaminated with methyl mercury or dioxin and is also the principal source of omega-3 fatty acids that reduces the risk of heart disease). Examples of pathogen/food combinations hint at the diversity of hazards (e.g., *Vibrio* in raw seafood, *Cyclospora* in berries). Chemical hazards include drugs, hormones, dioxin, and acrylamide. With the concerns over terrorism, food security has become a new and expensive federal priority. While agencies must address the microbial safety of processed foods, they also need to consider the role of these foods in epidemic obesity, which is rapidly becoming the leading cause of preventable death in the United States. Prioritizing these complex and sometimes conflicting public health problems requires data, judgment, and other tools of relative risk management.

## The Future

This report provides a brief regulatory perspective of some needs and challenges to assure the safety of the food supply. Experience indicates that new foodborne hazards will continue to emerge (*43-46*). At the time that this report is being written, antimicrobial resistant *Salmonella* strains including *S.* Newport and *S.* Typhimurium has become a new issue for which FSIS is actively considering a variety of outreach, enforcement, and regulatory options – all of which involve the allocation of new resources and infrastructure. Government must continually work with consumers and industry to enhance food safety. In order to respond to constantly evolving challenges, food safety agencies must have adequate resources, infrastructure and data to prioritize current and future food safety challenges. Appropriate levels of funding and recruitment are essential in order for food safety agencies to effectively respond to the increasing demands and expectations of the public and food industries for food safety assurance.

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## Chapter 20

# U.S. Food and Drug Administration's Imported and Domestic Produce Surveys

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Although the incidence of foodborne illnesses linked to fresh produce is low, over the last several years the proportion of foodborne illnesses associated with domestic and imported fresh fruits and vegetables has increased. This may be due, in part, to increased ability to detect foodborne illness outbreaks and to an increase in the consumption of fresh produce. To assist in the development of policy relating to produce safety, FDA has conducted a number of surveys since 1999 to gather data on the incidence and extent of pathogen contamination of selected produce, both imported and domestically produced. Produce items were selected based on criteria such as involvement in past foodborne illness outbreaks, structural characteristics, growing and processing conditions, and how they are consumed. The surveys focused on high-volume fresh produce including broccoli, cantaloupe, celery, cilantro, green onions, loose-leaf lettuce, parsley, strawberries, and tomatoes. Imported produce samples were collected at ports of entry, while domestic produce was collected at packing houses. Samples were analyzed for *Salmonella, Shigella,* and *Escherichia coli* O157:H7.

Although the incidence of foodborne illnesses linked to fresh produce is low, over the last several years the proportion of foodborne illnesses associated with domestic and imported fresh fruits and vegetables has increased. In January of 1997, President Clinton announced a Food Safety Initiative designed to improve the safety of the nation's food supply. The Department of Health and Human Services (DHHS), the Department of Agriculture (USDA), and the Environmental Protection Agency (EPA) sent a report to the President *(1)*, in May of 1997, that identified fresh produce as an area of concern. In October of 1997 *(2)*, President Clinton announced a plan entitled Produce & Imported Foods Safety Initiative to provide further assurance that fruits and vegetables consumed by the American public meet the highest health and safety standards.

The challenges in this area are self-evident. Most fresh fruits and vegetables are grown in non-sterile environments. Growers have less control over conditions in the field compared to an enclosed production facility. The surfaces of produce have natural microflora composed of microorganisms that are generally not significant to human health. However, low-level contamination of produce with pathogenic microorganisms may sporadically occur. Harvesting, washing, cutting, slicing, packaging and transporting offer opportunities for produce contamination. Most fresh produce is likely to be consumed raw without undergoing processes, such as cooking, that inactivate harmful microorganisms.

In October of 1998, the Food and Drug Administration (FDA) released a guidance document *(3)* entitled "Guidance for Industry - Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables." This document outlines good agricultural and good manufacturing practices (GAPs and GMPs) that reduce the risk of microbial contamination of fresh produce. This voluntary guidance does not impose unnecessary or unequal restrictions or barriers on either domestic or foreign products. Areas covered by the guide include water quality, manure management, worker training, field and facility sanitation, and transportation. Guidelines are set to control each potential source of contamination throughout the production process, from the farm to point-of-sale. In the absence of use of GAPs and GMPs, the risk of microbial contamination increases along with the likely extent of the contamination. Microbial safety hazards will be minimized if GAPs and GMPs are effectively implemented.

## Survey Design

To assist in the development of policy for the Produce & Imported Foods Safety Initiative, the FDA needed data on the incidence and extent of pathogen contamination on selected imported and domestic produce. In March of 1999, FDA initiated a 1000 sample survey *(4)*, the "Imported Produce Survey," focused on high-volume imported fresh produce. Eight produce items were

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selected for the survey: broccoli, cantaloupe, celery, cilantro, loose-leaf lettuce, parsley, scallions (green onions) and strawberries. These commodities were selected based on five risk factors: epidemiological outbreak data, structural characteristics, growing conditions, processing, and consumption. Collection of 125 samples was planned for each of the eight produce items. Tomatoes were added later in order to fulfill the assignment quota and culantro was added after it was determined to have a high rate of contamination. Produce was analyzed for *Salmonella*, *Shigella*, and *E. coli* O157:H7. Aerobic plate count (APC) and coliform analyses were planned but discontinued early in the assignment in order to reduce the time needed for analysis.

Due to a relatively elevated level of pathogens (i.e., *Salmonella* and *Shigella*) found on cilantro, culantro, and cantaloupe samples collected during the Imported Produce Survey, the FDA issued a follow-up survey in 2001 *(5)*, the "Follow-up Imported Produce Survey," focusing on collection and analysis of 300 samples of cantaloupe, cilantro, culantro, and tomatoes.

As a complement to the Imported Produce Survey conducted in 1999, the FDA issued the Domestic Produce Survey *(6)* in May of 2000. The study again focused on high-volume produce that is generally consumed raw. Eight commodities were selected for the domestic survey: cantaloupe, celery, cilantro, loose-leaf lettuce, parsley, scallions, strawberries, and tomatoes. Target collection areas were established so that each commodity was to be collected from regions that produce a significant portion of the crop.

Our purpose was not to attempt to detect every incidence of low-level, sporadic contamination but to detect those levels of contamination that might result from a failure to follow adequate GAPs and GMPs as specified by FDA guidance. The objectives of the produce surveys were to:

- collect and analyze samples of imported and domestic fresh produce to determine the incidence of microbial contamination on these commodities;
- undertake appropriate regulatory follow-up if violative samples were found, to protect U.S. consumers and foster corrective measures to implement practices to minimize microbial contamination on fresh produce; and
- obtain data to focus future research, risk assessment, industry training and food safety policy for the purpose of reducing foodborne illnesses resulting from contaminated fresh produce.

## Sample Collection and Analysis

Collection of imported produce at ports of entry was intended to reflect as many country/producer combinations as possible. Collection of domestic

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produce at packinghouses was carried out according to an FDA schedule specifying the produce items each district was to collect. Domestic samples were also collected from repackers and wholesalers, if the grower was identifiable.

All samples were collected aseptically and were shipped under refrigeration to the appropriate District's servicing laboratory. Samples were not frozen at any time prior to microbial analysis. Produce was prepared for microbial analysis in a manner that closely simulated minimal consumer preparations (e.g., visible dirt removed, stems and roots trimmed, outer leaves removed), but did not include a thorough wash step.

Each produce sample consisted of ten 16 oz., or 454 g, sub-samples that were each used to prepare a "sub-sample rinse" which required the sub-sample to be weighed and added to an appropriate amount of buffer solution to obtain a 1:1 ratio. The sample was agitated at 100 rpm for five minutes to release and distribute the bacteria from the surface of the produce into the buffer solution. Five of the 10 sub-samples were used to derive two composite samples for *Salmonella* analysis and the remaining five were used to derive two composite samples for *Shigella* analysis. The 10 sub-samples were also used to test for the presence of *E. coli* O157:H7. All commodities were analyzed for *Salmonella* and *E. coli* O157:H7. All commodities were analyzed for *Shigella* except cilantro, culantro, lettuce, and strawberries.

Isolates from each *E. coli* O157:H7 positive composite were subjected to pulsed field gel electrophoresis (PFGE) and ribotyping. Presumptive positives for *Salmonella* based on test kits were subjected to confirmation analysis as outlined in the Bacteriological Analytical Manual *(7)*, as well as, Most Probable Number (MPN) analysis *(8)*. Isolates from each *Salmonella* positive composite were serotyped and subjected to PFGE analysis and antibiotic resistance testing. *Shigella* analysis was done on a composite basis by polymerase chain reaction (PCR).

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## Imported Produce Survey

### Results

A total of 1003 samples were collected and analyzed from 21 countries during the survey. Countries that supply a greater amount of a specific produce item per year to the United States (e.g., cantaloupes from Mexico) were sampled more often than countries that supply a limited amount of the specified produce item (e.g., cantaloupes from Canada). Table I lists the countries that supplied produce samples for analysis, however, the identity of the countries from which contaminated produce was detected is not included.

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**Table I. Results of FDA's 1999 Imported Produce Survey**

| *Produce item* | *Number Analyzed* | *Number Positive* | *Percentage (%)*[e] | *Country of Origin*[ab] |
|---|---|---|---|---|
| Broccoli[c] | 36 | 0 | 0.0 | 3, 16 |
| Cantaloupe | 151 | 11 | 1.1 | 3, 6, 7, 8, 10, 11, 12, 16, 19 |
| Celery[d] | 84 | 3 | 0.3 | 3, 16 |
| Cilantro | 177 | 16 | 1.6 | 3, 5, 6, 16, 20, 21 |
| Culantro | 12 | 6 | 0.6 | 6, 21 |
| Lettuce[d] | 116 | 2 | 0.2 | 2, 3, 4, 9, 10, 13, 14, 15, 16, 17, 20 |
| Parsley[d] | 84 | 2 | 0.2 | 3, 5, 6, 13, 14, 16, 20 |
| Scallions | 180 | 3 | 0.3 | 3, 10, 13, 14, 16 |
| Strawberries | 143 | 1 | 0.1 | 1, 2, 3, 16, 18 |
| Tomatoes | 20 | 0 | 0.0 | 2, 16, 17 |
| Total | 1003 | 44 | 4.4 | ------ |

[a] Indicates that produce from that country was collected and analyzed but not necessarily contaminated.

[b] Numbers correspond to countries as follows:

1- Argentina
2- Belgium
3- Canada
4- Chile
5- Columbia
6- Costa Rica
7- Dominican Republic
8- Ecuador
9- France
10- Guatemala
11- Haiti
12- Honduras
13- Israel
14- Italy
15- Korea
16- Mexico
17- Netherlands
18- New Zealand
19- Nicaragua
20- Peru
21- Trinidad & Tobago

[c] Collection and analysis of broccoli was discontinued after 36 negative samples under the assumption that broccoli is frequently cooked, and the cooking process would be sufficient to destroy pathogens.

[d] Celery, lettuce, and parsley collections were discontinued before 125 samples were collected due to difficulty collecting the product in the assigned FDA district(s).

[e] (# positive /1003(total # sampled))*100

Of the 1003 samples, 4% were positive for pathogen contamination (i.e., *Salmonella* or *Shigella*), while 0% were contaminated with *E. coli* O157:H7. Table I shows the number of samples collected and analyzed and the number of confirmed positives for each produce item. The three produce items with the greatest incidence of pathogen contamination were cilantro, cantaloupe and culantro, accounting for 1.6, 1.1, and 0.6%, respectively, of the overall contamination (4.4%). The remaining produce items each contributed 0.3% or less to the overall contamination. Broccoli and tomatoes were not found to be contaminated and therefore did not contribute to the overall contamination level of 4.4%.

Table II shows the incidence of pathogen contamination of imported produce samples confirmed positive for *Salmonella* or *Shigella*. No samples were positive for *E. coli* O157:H7. Eighty percent of the contaminated samples were contaminated with *Salmonella*. The incidence of *Salmonella* on the total number of produce items sampled was 3.5% and the incidence of *Shigella* was 0.9%.

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**Table II. Pathogen Contamination of Imported Produce**

| *Produce Item* | *Analyzed* | *Positive* | *Salmonella* | *Shigella*[a] |
|---|---|---|---|---|
| Broccoli | 36 | 0 | ----- | ----- |
| Cantaloupe | 151 | 11 | 8 | 3 |
| Celery | 84 | 3 | 1 | 2 |
| Cilantro | 177 | 16 | 16 | ----- |
| Culantro | 12 | 6 | 6 | ----- |
| Lettuce | 116 | 2 | 1 | 1 |
| Parsley | 84 | 2 | 1 | 1 |
| Scallions | 180 | 3 | 1 | 2 |
| Strawberries | 143 | 1 | 1 | ----- |
| Tomatoes | 20 | 0 | ----- | ----- |
| Total | 1003 | 44 | 35 | 9 |

[a] Only samples of cantaloupe, celery, parsley, scallions, and tomatoes were analyzed for the presence of *Shigella*, except that one sample of loose-leaf lettuce was analyzed for *Shigella* upon request of the collection district. Two composite positives were found and reported.

*Salmonella* on produce may have been the result of contamination from human contact, such as unsanitary food handlers, or from environmental sources. *Salmonella* is widespread in poultry and swine and is often found in water, soil, animal feces, and on food contact surfaces. This list is not inclusive, however, and the contamination might have been derived from another source. *Shigella* is transmitted via the fecal-oral route and would likely be transmitted by unsanitary handling by infected food handlers. This microorganism is also found in water polluted with human feces.

# Regulatory Follow-up

## Detention Without Physical Examination

When a product/grower combination was found to be violative (i.e., the produce tested positive for *Salmonella or Shigella*), subsequent shipments were either detained without physical examination (DWPE)[1] or sampled as "suspect" products under the appropriate compliance program. FDA did not approve or recommend reconditioning of detained products; all detained products were destroyed.

Confirmed presence of *Shigella* on produce samples is considered to be a correctable human failure to follow GAPs and GMPs (i.e., human fecal contamination) and represents a potentially significant health hazard. Therefore, it was recommended that the current shipment and subsequent shipments would be detained and the grower/shipper be placed on DWPE after one positive composite was found.

Confirmed presence of *Salmonella* on produce samples may be due to a chance event (e.g., wildlife in field) and would not necessarily indicate poor agricultural practices. However, more than a single sporadic positive could be an indicator of poor practices. Therefore, for samples found with one composite positive for *Salmonella*, the shipment yielding the positive sample was refused admission into the U.S. and the next 10 shipments of that product from that grower/shipper were sampled. If a second positive was detected, the shipper/grower was recommended for DWPE. If a second positive was not detected among the next ten shipments, intensified sampling was discontinued. If two positive samples for *Salmonella* were found in the same entry, the shipper/grower would be placed on DWPE.

If the grower of the contaminated produce was identifiable (e.g., through records or labeling), a farm inspection by FDA could be initiated. If a farm inspection was refused or if the findings of the inspection were indicative of unsanitary practices that would lead to microbial contamination, then the grower was placed on DWPE. It was not FDA's intent to conduct farm inspections in all

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[1] DWPE means that an import shipment is refused entry into U.S. commerce unless the importer presents evidence, e.g., test results, to FDA showing that the item meets U.S. safety requirements. DWPE can be imposed when violative findings for a grower/shipper are of a nature that suggest that future shipments from that grower/shipper may also be violative. DWPE is imposed to protect consumers from potentially contaminated subsequent shipments from that grower/shipper until the firm implements appropriate corrective measures.

cases where DWPE was imposed, but to do so in some cases for information gathering purposes.

Twenty-one firms were placed on DWPE and four FDA-initiated farm inspections were conducted as follow-up. Examination of facilities and observation of standard practices helped to identify sources of contamination and possible corrective actions. Likewise, review of packages submitted by firms requesting removal from DWPE provided useful information about potential sources of contamination and successful corrective actions.

Of the 21 implicated firms, 7 firms were placed on DWPE due to the presence of *Shigella* in one composite of cantaloupe, celery, lettuce, or scallions, while 14 firms were placed on DWPE due to the presence of *Salmonella* in two composite samples of cantaloupe, celery, cilantro, or culantro.

Based on farm investigations and other information, a failure to follow GAPs and GMPs was often associated with the findings of pathogen contamination. In particular, inadequate manure management and lack of appropriate field and transport sanitation practices was most frequently associated with contamination. Specific problems included fields that were open to domestic animals and were fertilized by untreated animal manure, equipment and tools not being sanitized, unsanitary harvesting and/or packing equipment (e.g., woven plastic bags to collect culantro after harvest) and packing practices, and unsanitary methods of transportation (e.g., trucks washed with non-chlorinated water and/or cleaned infrequently). In at least one instance, a firm placed on DWPE could not provide documentation to certify the cleanliness of the water used for irrigation and fertilization.

## Removal from DWPE

FDA made the decision to remove firms from DWPE based on information packages submitted by the firms that documented the corrective actions taken to remove the conditions producing the violations.

All firms removed from DWPE identified, implemented and verified all identifiable corrective actions. Corrective measures included fencing areas to eliminate entry by animals, cessation of use of untreated animal manure as fertilizer, food safety training for employees involved in the harvest and/or the maintenance of harvest equipment used in the field, replacement of woven plastic harvesting bags with easily cleanable plastic crates, and increased frequency of truck cleaning with a sanitizer (e.g., chlorine) added to the cleaning water. Proactive measures focused on implementing GAPs and GMPs to ensure a safer food supply for export.

As a consequence of this survey, food safety training was implemented at firms placed on DWPE for harvest workers to teach proper practice for

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harvesting and maintaining the harvest equipment in a sanitary manner. Successful implementation of food safety training was a factor in the removal of some firms from DWPE. At least two firms removed from DWPE voluntarily implemented changes that followed GAPs outlined in the guide (e.g., training on worker health and hygiene, sanitation of fields and packing facilities, and manure management).

### Follow-up Imported Produce Survey

During the follow-up imported produce survey of 2001, a total of 257 samples of cantaloupe, cilantro, culantro, and tomatoes were collected and analyzed. One cilantro sample was positive for *Salmonella* contamination and one tomato sample was positive for *Salmonella* contamination. None of the samples were positive for *Shigella* or *E. coli* O157:H7. Samples were taken from 10 countries: Belgium, Costa Rica, Dominican Republic, Guatemala, Honduras, Israel, Mexico, Netherlands, Nicaragua, and Spain. The results of this survey are presented in Table V later in this chapter.

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## Domestic Produce Survey

### Results

A total of 1028 domestically produced samples from 18 different states were collected; see Table III for a list of states from which a particular commodity was collected.

Of 1028 samples collected and analyzed, 11 (1.1%) of the samples were positive for pathogen contamination (i.e., *Salmonella* or *Shigella*). *E. coli* O157:H7 was not found on any of the domestic produce samples. Table III shows the number of samples collected and analyzed and the number of confirmed positives for each produce item. One or more samples of cantaloupe, cilantro, lettuce, parsley, and scallions were positive for pathogen contamination, while celery, strawberry, and tomato samples were not contaminated. Cantaloupes had the highest number of positive samples (5), followed by scallions (3), cilantro, lettuce and parsley (1 each).

**Table III. Results of FDA's 2000 Domestic Produce Survey**

| *Produce Item* | *Number Analyzed* | *Number Positive* | *Percentage (%)* | *State of Origin*[a] |
|---|---|---|---|---|
| Cantaloupe | 164 | 5 | 0.5 | 2, 3, 4, 5, 6, 7, 10, 11, 13, 15, 16, 17, 18 |
| Celery | 120 | 0 | 0.0 | 3, 4, 5, 9, 13, 7 |
| Cilantro | 85 | 1 | 0.1 | 3, 5, 6, 13 |
| Lettuce | 142 | 1 | 0.1 | 2, 3, 4, 5, 6, 12, 13 |
| Parsley | 90 | 1 | 0.1 | 2, 3, 5, 6, 12, 13, 14 |
| Scallions | 93 | 3 | 0.3 | 2, 3, 4, 5, 6, 11, 13, 14, 16, 17 |
| Strawberries | 136 | 0 | 0.0 | 3, 5 |
| Tomatoes | 198 | 0 | 0.0 | 1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 13, 15, 16 |
| Total | 1028 | 11 | 1.1 | ------ |

[a] Indicates that produce from that state was collected and analyzed but not necessarily contaminated

1- Alabama
2- Arizona
3- California
4- Colorado
5- Florida
6- Georgia
7- Iowa
8- Louisiana
9- Michigan
10- Maryland
11- North Carolina
12- New Jersey
13- New York
14- Ohio
15- Oklahoma
16- South Carolina
17- Texas
18- Virginia

Table IV shows the incidence of pathogen contamination on produce samples confirmed positive for either *Salmonella* or *Shigella* contamination. Of the 11 confirmed positive domestic produce samples, six samples were positive for *Salmonella* and five samples were positive for *Shigella*.

## Regulatory Follow-up

Regulatory follow-up for the Domestic Produce Survey was consistent with that of other domestic FDA programs and/or assignments. For all 11 violative samples that were found, the FDA determined whether any portion of the lot from which the contaminated sample came was still in commerce. In all cases, since the product was a fresh produce item with a short shelf-life, the entire lot had been sold and consumed or discarded. Therefore, the product was not available for voluntary recall by the violative firms.

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**Table IV. Pathogen Contamination of Domestic Produce**

| *Produce Item* | *No. Analyzed* | *No. Positive* | *Salmonella* | *Shigella*[a] |
|---|---|---|---|---|
| Cantaloupe | 164 | 5 | 4 | 1 |
| Celery | 120 | 0 | ----- | ----- |
| Cilantro | 85 | 1 | 1 | N/A |
| Lettuce | 142 | 1 | 1 | N/A |
| Parsley | 90 | 1 | 0 | 1 |
| Scallions | 93 | 3 | 0 | 3 |
| Strawberries | 136 | 0 | ----- | N/A |
| Tomatoes | 198 | 0 | ----- | ----- |
| Total | 1028 | 11 | 6 | 5 |

[a] Only samples of cantaloupe, celery, parsley, scallions, and tomatoes were analyzed for the presence of *Shigella*.

The presence of violative samples led state officials to initiate farm investigations and the FDA to perform three follow-up farm investigations. The state investigators and the FDA officials found no specific problem areas on the farms that could be identified as the source of the contamination. However, this is not unusual as environmental and other production factors may change between the time contamination occurs and when investigators reach a farm. The investigations allowed officials to examine farm facilities and observe standard practices to help identify sources of contamination and possible appropriate corrective actions.

Table V shows the produce items analyzed during each of the three FDA surveys, the number of samples positive for either *Salmonella* or *Shigella* (none were positive for *E. coli* O157:H7 in any survey), and the percentage of each produce item that was violative of the total number of samples collected during each survey.

Certain produce items were found to be contaminated in all three FDA initiated produce surveys. In particular, cantaloupe, cilantro, culantro, and scallions were found to be contaminated in larger percentages than would be expected due to low-level, sporadic contamination. More than 7% of imported cantaloupes that were analyzed during the initial survey were found to be contaminated while 3% of domestically produced cantaloupes were contaminated. *Salmonella* was the pathogen most often found on the cantaloupes. Structural characteristics of cantaloupes, e.g., the webbing on the outer surface of the fruit may provide a suitable environment for bacteria to attach and survive. Nine percent of imported cilantro samples that were analyzed were found to be contaminated in the initial survey; the potential for cilantro to be a vehicle for foodborne illness was further supported when 3.3% of imported samples were found to be contaminated in the follow-up study of imported produce. Domestically produced cilantro was contaminated in one of 85 samples. In all cilantro samples, *Salmonella* was the confirmed pathogen. Similarly, although the sample size for culantro was small, 6 of 12 imported samples were found to be contaminated, again with *Salmonella*. Scallions, both imported and domestically produced, were found to be contaminated. Interestingly, only one imported scallion sample was contaminated with *Salmonella*; the other two imported scallion samples and all three domestic scallion samples were contaminated with *Shigella*.

**Table V. Results of FDA Fresh Produce Surveys 1999-2001**

| | *Imported Produce Survey (n=1003)* | | *Follow-up Imported Survey (n=257)* | | *Domestic Produce Survey (n=1028)* | |
|---|---|---|---|---|---|---|
| *Produce Item* | *Number Analyzed* | *Number Positive (%[a])* | *Number Analyzed* | *Number Positive (%[a])* | *Number Analyzed* | *Number Positive (%[a])* |
| Broccoli | 36 | 0 (0.0) | n/a | n/a | n/a | n/a |
| Cantaloupe | 151 | 11 (7.3) | 51 | 0 (0.0) | 164 | 5 (3.0) |
| Celery | 84 | 3 (3.6) | n/a | n/a | 120 | 0 (0.0) |
| Cilantro | 177 | 16 (9.0) | 30 | 1 (3.3) | 85 | 1 (1.2) |
| Culantro | 12 | 6 (50.0) | 7 | 0 (0.0) | 0 | 0 (0.0) |
| Lettuce | 116 | 2 (1.7) | n/a | n/a | 142 | 1 (0.7) |
| Parsley | 84 | 2 (2.4) | n/a | n/a | 90 | 1 (1.1) |
| Scallions | 180 | 3 (1.7) | n/a | n/a | 93 | 3 (3.2) |
| Strawberries | 143 | 1 (0.7) | n/a | n/a | 136 | 0 (0.0) |
| Tomatoes | 20 | 0 (0.0) | 169 | 1 (0.6) | 198 | 0 (0.0) |

[a] (# positive/# sampled) × 100%.

FDA's initial produce surveys show the potential for pathogen contamination on fresh produce, which may impact the safety of the product that reaches the consumer. Adequate GAPs and GMPs are needed at the farm level to minimize the risk of microbial hazards to the consumer. FDA will continue to conduct these types of surveys as further studies with increased numbers of commodities analyzed would prove useful in determining specific commodities to address, and perhaps, specific risk factors in an operation that might contribute to the contamination of fresh produce.

The imported and domestic produce surveys were designed to provide data to FDA on the incidence and extent of pathogen contamination on selected domestic and imported produce. This information is needed in order to develop policy and guidance for the Produce & Imported Foods Safety Initiative and to focus education/outreach efforts. The intent was not to draw quantitative

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comparisons between the incidence of contamination of domestic and imported produce. A larger sample size is needed in order to make such comparisons. However, the surveys were designed to allow the agency to make some qualitative comparisons in order to better understand the potential risks associated with select produce items.

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## Chapter 21

# Food as a Weapon of Terrorism

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While food is an attractive vehicle for a terrorist biological weapon, the use of food as a weapon may be more difficult than initially appears. The events of September 11, 2001, and the subsequent anthrax incidents gave rise to concerns about unconventional terrorist attacks, including a similar threat on the U.S. food supply. In the aftermath of those incidents, the Food and Drug Administration (FDA) took steps to improve its ability to prevent, prepare for, and respond to incidents of food contamination. Though motivated by the concerns about deliberate contamination, those activities built upon and expanded the agency's continuing efforts to protect consumers from foods that have been unintentionally contaminated. As part of those activities, FDA assessed the risk to and vulnerability of the U.S. food supply to an act of terrorism. Clearly some foods are not very susceptible to deliberate contamination.

U.S. government work. Published 2006 American Chemical Society

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## Historical Perspective

Food has been used as a weapon since ancient times. The polluting of wells and water supplies with corpses of both men and animals is probably as old as history. Romans and later armies during the Middle Ages were known to put dead animals into the water supplies of their enemies with the intent of at least weakening and demoralizing them (*1*).

One of the first recorded uses of contaminated food (water) as a weapon was in 600 BC when Solon, the legislator of Athens, used hellebores—hellebore is a toxic plant—to contaminate the River Pleithenes. Drinking from the river gave the defenders of Kirrha explosive diarrhea making them unable to fight. The Athenians then won a subsequent victory.

Then, around 200 BC, the Carthaginians did poorly in a battle and before their retreat, they left behind wine they had poisoned with mandragora, a root that contains a narcotic. Their enemy drank the wine, the narcotic took affect, they fell into a sleep, and the Carthaginians came back and slaughtered them all while they were unconscious (*2*).

Food was used as a weapon in North America in 1623 when the Jamestown colonists invited an Indian leader named Chiskiack, along with his family and some two hundred members of his tribe, for treaty talks and a feast. British negotiators of the treaty with Indians near the Potomac River, under Chief Chiskiack, offered a toast "symbolizing eternal friendship." The British had poisoned the food. The Indians (chief, family, advisers, and two hundred in the retinue), then died immediately of poisoning! (*3*).

Some territorial newspapers in the Old West advised settlers on how to leave strychnine-laced food near Indian trails so that Indians could be exterminated. In Minnesota, the Winona Daily Republican announced on September 24, 1863: "The State reward for dead Indians has been increased to $200 for every red-skin sent to Purgatory. This sum is more than the dead bodies of all the Indians east of the Red River are worth." It was rumored that Minnesota settlers left poisoned cakes behind when fleeing the territory during the Indian war. More attempted food poisoning incidents occurred in the West in struggles between miners and Indians during and after the Gold Rush (*4*).

During the American Civil War, Confederate troops led animals to ponds where they shot them and left their bodies to rot in the water so as to deny Sherman's advancing army any potable water supplies as it made its way to the sea. Northern troops were constantly warned about the possibility of eating intentionally poisoned food obtained from Southern sources (*5*).

During World War I the Germans attempted to spread livestock diseases, such as glanders and anthrax, among horses, mules, sheep, and cattle to upset the Allies food supplies. In World War II horses and mules were still used to transport supplies along the battle fronts. Anything that killed or maimed the

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animals offered a tactical advantage. However, the attempt had little success. In non-food related biological warfare during WW II the Japanese, who were not signatories to the Geneva Protocol, killed as many as 10,000 people in Manchuria while developing various disease agents, including anthrax, cholera, typhoid, and plague. Later, during the war in China, hundreds of thousands of Chinese civilians suffered from these diseases as Japanese aircraft dropped paper bags filled with plague-infested fleas over the cities of Ningbo and Quzhou in Zhejiang province. In food related attacks they contaminated wells and, just as the English and Americans did to Indians, they distributed poisoned foods.

More recent history on deliberate food contamination will be discussed in the following sections.

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## Risk Assessment

The events of September 11, 2001, and the subsequent anthrax incidents (*6*) gave rise to concerns about unconventional terrorist attacks, including the threat of attacks on the U.S. food supply. Those events also heightened international awareness that nations could be targets for biological or chemical terrorism--a threat that had long concerned military and public health officials.

In the aftermath of those incidents, the FDA took steps to improve its ability to prevent, prepare for, and respond to incidents of food contamination. Though motivated by the concerns about deliberate contamination, those activities built upon and expanded the agency's continuing efforts to protect consumers from foods that have been unintentionally contaminated (e.g., through processing failures or handling errors).

As part of those activities, FDA assessed the risk to, and vulnerability of the U.S. food supply to an act of terrorism (*7*). However, most of those assessments contain classified information. To promote transparency, FDA prepared a publicly available assessment of the risks to public health of a terrorist attack on the food supply and of serious illness due to unintentional food contamination. This Risk Assessment follows the generally accepted framework for risk assessments endorsed by the Codex Alimentarius Commission, the U.S. National Academy of Sciences, and other authoritative bodies (*8*). The framework divides risk assessment into four components: (1) hazard identification, (2) hazard characterization (or dose-response assessment), (3) exposure assessment, and (4) risk characterization. Unlike traditional risk assessments, however, which focus on one hazard, this assessment addresses the broad range of hazards available to terrorists intending to contaminate food, as well as hazards that accidentally are introduced into food.

FDA concluded that though the likelihood of a biological or chemical attack on the U.S. food supply is uncertain, significant scientific evidence documents the risk to public health from food that has been unintentionally contaminated (*9*). Notwithstanding the uncertainties described in the risk assessment, and given the broad range of agents that may contaminate the food supply that FDA regulates, the agency concluded that there is a high likelihood, over the course of a year, that a significant number of people may be affected by an act of food terrorism or by an incident of unintentional food contamination that can result in serious foodborne illness.

## Deliberate Versus Accidental Contamination

Pathogens that historically have been linked to unintentional food contamination, such as *E. coli* O157:H7 and *Salmonella* spp., were identified by the CDC as "critical" agents for food terrorism (*10*). However, the risk to consumers and the public health response to these known pathogens would be comparable, regardless of whether the contamination was deliberate or accidental. Officials responding to a foodborne illness outbreak probably would not know whether the contamination was accidental or intentional until an investigation was performed to determine the source of the outbreak. Even then, officials might never be able to conclusively determine whether the food was deliberately or inadvertently contaminated.

Acts of deliberate food contamination have already occurred in the U.S. In 1984, for example, the members of a religious cult contaminated salad bars with *Salmonella typhimurium* in order to disrupt a local election. This incident caused 751 cases of salmonellosis and resulted in the hospitalization of 45 of the victims (*11*). It should be noted that an attempt by the cult to contaminate produce in a grocery store was largely unsuccessful. Also the outbreak was initially ascribed to unintentional contamination by food handlers and not to deliberate contamination. No one then suspected bioterrorism. In a more recent incident, in May 2003, a supermarket employee pleaded guilty to intentionally poisoning 200 pounds of ground beef with an insecticide containing nicotine. Although the tainted meat was sold in only one store, 111 people, including approximately 40 children, were sickened (*12*). Nicotine is used in a limited number of pesticides because of its toxic properties. Again it should be noted that unintentional contamination of food by chemicals occurs sporadically, including reports of contamination by pesticides.

Perhaps some of the most egregious examples of deliberate food contamination that recently have occurred in other countries have been reported in China. In September 2002, 40 people died and 300 were sickened near Nanjing, China after the owner of a fast-food outlet poisoned a competitor's breakfast foods with rat poison (*13*). One year earlier, 120 people in China were sickened when the owners of a noodle factory reportedly laced their food with

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(presumably) the same kind of rat poison (*14*). The suspected rat poison called "dushuquiang" is an arsenic-based poison which is highly toxic and long-lasting, and apparently, too readily available in China. China has announced that it will severely punish those who engage in the illegal making, buying and selling, transporting or selling of "dushuquiang". They also said that they will consider it very serious if the use of the poison results in the death of more than three people or their serious wounding. As evidenced by their actions there seems to be little doubt on the part of Chinese officials that these poisonings were deliberate. It is possible that these saboteurs did not realize the severity of the acts that they were committing and thought that the effects of the added poisons might sicken a few people at the worst. These poisonings do illustrate the potential severity of adding chemical poisons to foods and perhaps also illustrate some of the uncertainties encountered when deliberately contaminating foods.

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## Possible Agents for Food Terrorism

The incidents discussed above illustrate just a few of the many possible agents for food terrorism. The range of such agents is broad, and their characteristics varied; they may include:

- Biological and chemical agents;
- Naturally occurring, antibiotic-resistant, and genetically engineered substances;
- Deadly agents and those tending to cause gastrointestinal discomfort;
- Highly infectious agents and those that are not communicable;
- Agents that can be weaponized.

Before the September 11th attacks, the U.S. Centers for Disease Control and Prevention (CDC) had already developed a strategic plan on biological and chemical terrorism. The CDC plan identified and ranked several foodborne pathogens as critical agents for possible terrorist attacks. Among the high-priority biological agents ("Category A" agents) were *Bacillus anthracis* (anthrax) and *Clostridium botulinum* (botulism), both of which are deadly pathogens and may contaminate food. Most of the foodborne biological agents identified by CDC were classified as "Category B" agents because they are moderately easy to disseminate and cause moderate morbidity and low mortality. The Category B biological agents include *Salmonella* spp., *Shigella dysenteriae, E. coli* O157:H7, and ricin (*10*). Notably, several of the pathogens identified by CDC as critical biological agents also are known to pose a significant threat due to unintentional contamination of food (*14*).

In addition, the CDC identified certain chemicals as possible agents for a terrorist attack. Those included heavy metals, such as arsenic, lead, and mercury, and pesticides, dioxins, furans, and polychlorinated biphenyls (PCBs), all of which may be used to contaminate food (*10*). These toxins may also be introduced inadvertently into foods and linked to human health effects (*15*).

The CDC further warned that terrorists might use combinations of these agents, attack in more than one location simultaneously, use new agents, or use organisms that are not on the critical list (e.g., common, drug-resistant, or genetically engineered pathogens) (*16*).

Generally harmful agents that might be used in food by terrorists fall into two general groups: those substances readily available to any individual and those that are more difficult to acquire. If history is any indicator, substances that are readily available are usually favored.

In early September 2003, the U.S. Federal Bureau of Investigation (FBI) issued a bulletin warning that terrorists might use two naturally occurring toxins, nicotine and solanine, to poison U.S. food or water supplies. The FBI noted that terrorist manuals and documents recovered in Afghanistan refer to the use of these substances as poisons. Citing the supermarket employee that deliberately contaminated ground beef with an insecticide containing nicotine, FBI officials advised: "Such lone offenders, whether al-Qaida [*sic*] sympathizers or domestic criminals, are a concern to FBI because they are so difficult to detect" (*17*).

If an unintentional contamination of one food, such as clams (*18*), can affect 300,000 individuals, a concerted, deliberate attack on food could be devastating, especially if a more dangerous chemical, biological, or radionuclear agent were used. It would be reasonable to assume that a terrorist using the food supply as a vehicle for attack would use an agent that would maximize the number of deaths associated with the contamination (*19*). Many of these agents are the same pathogens that have, in the past, been linked to significant outbreaks of foodborne illness due to unintentional contamination.

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## Vulnerability of the Food Supply

Before 2001, there was little awareness of the vulnerability of the U.S. food supply to terrorism. We knew about intentional contaminations and tampering but were not thinking that an enemy might want to harm large numbers of people through contaminating the food supply.

In 2001, the Department of Defense released **The Threat and Response Report** that identified foods in agriculture as a critical infrastructure. Then, more recently the White House, in the **National Strategy for Physical Protection of Critical Infrastructures and Key Assets**, officially designated foods as a part of critical infrastructure.

Prior to September 2001, the FDA had a contract with the Battelle Memorial Institute to do a vulnerability assessment with specific foods and agents. They provided a decision-making tool so that, in the event of an outbreak, there would be a logical way to trace back to determine the most likely agents involved. After September 2001, to accomplish an internal vulnerability assessment of the food supply, FDA CFSAN began using a technique known as operational risk management (ORM) which is a systematic tool for this evaluation.

Six steps were used in this particular operational risk management (Figure 1).

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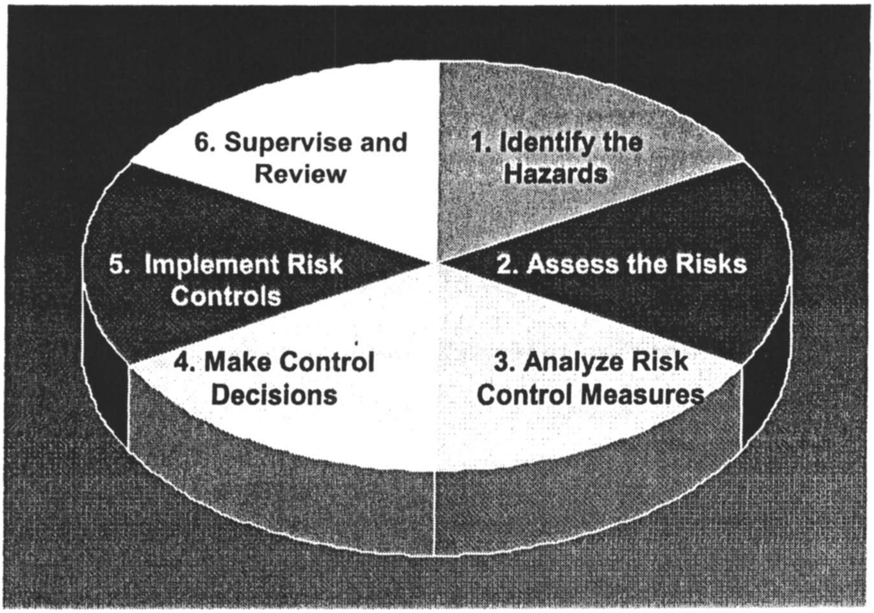
1. Identify the Hazards
2. Assess the Risks
3. Analyze Risk Control Measures
4. Make Control Decisions
5. Implement Risk Controls
6. Supervise and Review

*Figure 1. The six steps of Operational Risk Management*

Step 1 in ORM is to identify the hazard(s) that are of concern if a specific food were tampered with or sabotaged. The second step is to assess what are the corresponding risks. The rest of the ORM process deals with the control measures and their implementation. It was quickly realized that the risk assessment step (#2) is highly dependent on the agent employed and the type of food contaminated.

In order to evaluate the risk from a particular contaminated food, two factors were evaluated from a public health standpoint. One was the severity of an attack in terms of human illnesses. This could range anywhere from negligible or minor public health incidents to catastrophic events where hundreds were sickened or died. The other was the probability that this food product would be contaminated with that agent. This probability could range anywhere from unlikely to frequent. Agents that would be hard to acquire perhaps combined with foods with limited access or potential to be a vector for the agent would be classified as Unlikely (Figure 2). Frequent would define instances where agents have occurred in foods, that have caused illnesses and death, and could be added to these foods again. The risk is then calculated by giving each of these factors a numerical value and then combining the two as shown in Figure 2.

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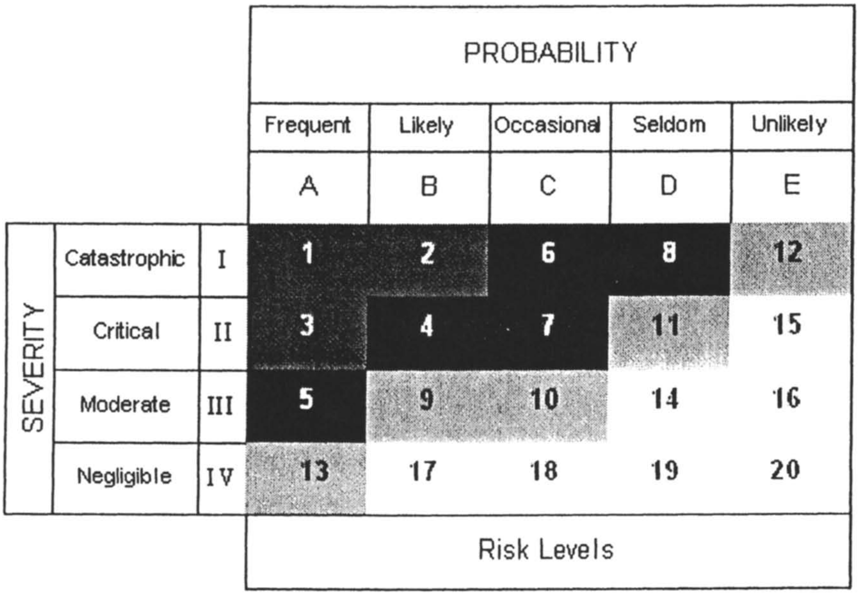

| | | | PROBABILITY | | | | |
|---|---|---|---|---|---|---|---|
| | | | Frequent | Likely | Occasional | Seldom | Unlikely |
| | | | A | B | C | D | E |
| SEVERITY | Catastrophic | I | 1 | 2 | 6 | 8 | 12 |
| | Critical | II | 3 | 4 | 7 | 11 | 15 |
| | Moderate | III | 5 | 9 | 10 | 14 | 16 |
| | Negligible | IV | 13 | 17 | 18 | 19 | 20 |

Risk Levels

*Figure 2. Calculation of risk*

A separate calculation is done for each agent and specific food or activity. This allows a separation of the food, the agent and the activity into scenarios such as high, medium and low. This is a rather coarse tool for evaluating risk, but it allows concentration on foods that may be used in attacks.

## Food as a Vehicle for Terrorist Agents

To conduct the risk assessment described above, knowledge of food technology and food science along with food microbiology and food chemistry is needed. It is one thing to have an agent and put it into a food. But, in many cases, we know that it will not be able to survive the environment or food matrix or processing conditions that would normally be applied to that food. The agent might also undergo dilution, separation or inactivation during the handling and storage of the food product. Additionally, the food product might not be consumed if its texture, taste, smell or visual appearance is altered by the agent.

FDA CFSAN was so impressed with the original internal risk assessments by the above method that it believed that a second more comprehensive look was necessary and that this approach needed to be validated. CFSAN then commissioned a team put together by the Institute of Food Technologists (IFT) which included food microbiologists, food chemists, toxicologists, and forensics experts, from the industry, government, and universities who had expert knowledge of foods, including food processing, to evaluate this approach and perform an independent risk ranking. Using the same foods, the same agents, and the same processes the expert committee came up with virtually the same rankings. Because they had more knowledge on some products and information on some additional food products and processes, they identified some additional vulnerabilities. So the IFT committee confirmed our ability to rank these hazards and evaluate them in terms of potential risks of deliberate contamination.

These assessments were limiting in that they only considered morbidity and mortality of a terrorist attack on the food. They didn't consider the economic consequences, public alarm, and loss of confidence in, or interruption of, the food supply. To assess more completely the risks that might be associated with a particular food, CFSAN has assembled other tools to assess these and other consequences and integrated them with public health concerns. Known as CARVER + Shock, this process independently rates seven factors that affect the desirability of a food as a terrorist target.

While analyses of risk were useful to prioritize foods as potential targets for terrorists, they also were invaluable in showing the knowledge gaps we had regarding the behavior of these agents in our foods. The FDA and food industry needed to know how new or non-traditional agents that might be used in bioterrorism activities behave in foods. There are a number of characteristics of foods and changes to foods during processing that can alter an agent's survivability if it's a living biological agent or alter its toxicity if it is a poison. This was also realized by the IFT in their recent report to the FDA on ***Preventative Controls Needed to Minimize/Reduce the Risk of an Intentional Act of Terrorism and/or Contamination***. General questions that still need to be answered are: What is the effect of the food and also food processing on these

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new or added unconventional agents? Can we expect to get some protection from the food processes as they now exist in the industry? Currently, information is limited on the behavior of potential bioterrorism agents in foods.

## Research at the National Center for Food Safety and Technology (NCFST)

Dealing with microorganisms and toxins in foods is not new for the food industry. Thermal and other processes are used everyday in the food industry to inactivate toxins and pathogens. Food security issues are also not new for the food industry because it historically has had the possibility of tampering and/or the counterfeiting of food products.

The NCFST was in a good position to rapidly change some of its research focus to address concerns about processing effects to some of these unconventional bioterrorist (BT) agents that might be introduced to our foods. As part of its interface with the industry, the NCFST can suggest the kinds of prevention measures that might be effective. Also research on prevention measures and transfer of this research to the industry is important. NCFST, through its collaborative efforts, arrived at this program collectively with FDA, industry and with academic input. It focused on intervention and prevention strategies that include Counter Terrorism (CT) and Biological Safety level (BSL) pilot-plant research. It is also investigating new technologies in food processing and packaging that may inactivate bioterrorist agents in our foods.

There are three ongoing projects. One is on the survival and growth of nontraditional pathogens in foods. The second project is on the thermal resistance of microbial agents that might be associated with bioterrorism. Third, the NCFST has a project on the evaluation of ELISA assays to detect *Clostridium botulinum* toxins in foods. As mentioned before, there is really not much of a knowledge base on some of these pathogens and their behavior in foods. There are various natural components of foods that may inactivate or neutralize bioterrorism agents. We need to know about how food composition affects BT agents.

The NCFST has been working with organisms that do not require a CDC Select Agent license. Until a select agent license for some of these organisms can be acquired, work with microorganisms which are surrogates should suffice. The objective is to determine if the unconventional organism that might be introduced into a food will survive, grow or gradually die. It is anticipated that this information will help in FBI or FDA Case investigations in the event of a suspected attack with a specific agent and will also aid public health officials in risk management.

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So far, we are finding that some of these agents are, indeed, poor growers in nutrient-limited foods. We are also finding that some of the biological agents that contain virulence genes die off more quickly in foods.

The NCFST will look at the behavior of these unconventional organisms in other shelf-stable foods like infant formula, juices, sports drinks, et cetera.

NCFST is also studying how some of our traditional food processing affects these agents. One project is investigating the thermal resistance of these unconventional organisms with common food-processing techniques such as pasteurization or extrusion and/or some of the other unit processes that might be used within the food industry. A project of interest is the thermal resistance of nontraditional microbial agents such as *Clostridium botulinum* toxin. Also of interest is the combination of the effects of heat, pH, salt and a number of other parameters along with the processing of foods on the biological activities of added agents and toxins. We have very little data on how some of these toxins behave and react as they progress through food-processing operations.

It is important to correlate our biochemical or agent test results with actual loss of biological activity. We realize, from our past work with allergens, that we have to look at the behavior of ELISA test kits to determine that they are truly valid for allergenicity assessments. This same criterion is true for biological activity. We know, for example, that botulinum toxin is still present (but inactive) after processing the food. So we need to make sure that we are actually following true biological activity of the agent in our food process assessments and not just following the inactivated protein.

If there is a terrorism incident involving contamination of a food processing plant, the food processing plant is going to be a liability until it is decontaminated. Rapid decontamination of a the food facility would greatly lessen the impact of a terrorist attack. We may sterilize the food and bury it in a landfill, but we are certainly going to have to decontaminate the food plant and equipment at the site where it exists.

Sanitation is not new to in the food industry. We need to know how effective the currently used sanitizers are against these unconventional pathogens. While we are investigating the effects of our processing on these agents, we can concurrently investigate decontamination of food processing equipment and facilities.

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## The Need for Improved Research Facilities and Protective Measures

To conduct food processing research on potential bioterrorism agents requires upgrades of pilot plants and laboratories. To conduct food pilot-plant-related research on these agents, we will have some unique capabilities; that is, we are going to have a Biological Safety Level (BSL) 3 pilot plant (one in which we can protect scientists against pathogens) and we will meet the recent new Select Agent requirements of the Patriot Act.

There are BSL laboratories in the United States, but very few BSL3 food pilot plants. There are none that meet all BSL3 and Patriot Act requirements. So we have a lot of work to do before we can conduct some of the research described above. To readily transfer select agents between the pilot plant and laboratory requires much more stringent personal protection and security precautions than previously.

A schematic of the modified pilot-plant and laboratory design similar to one that is required is shown below:

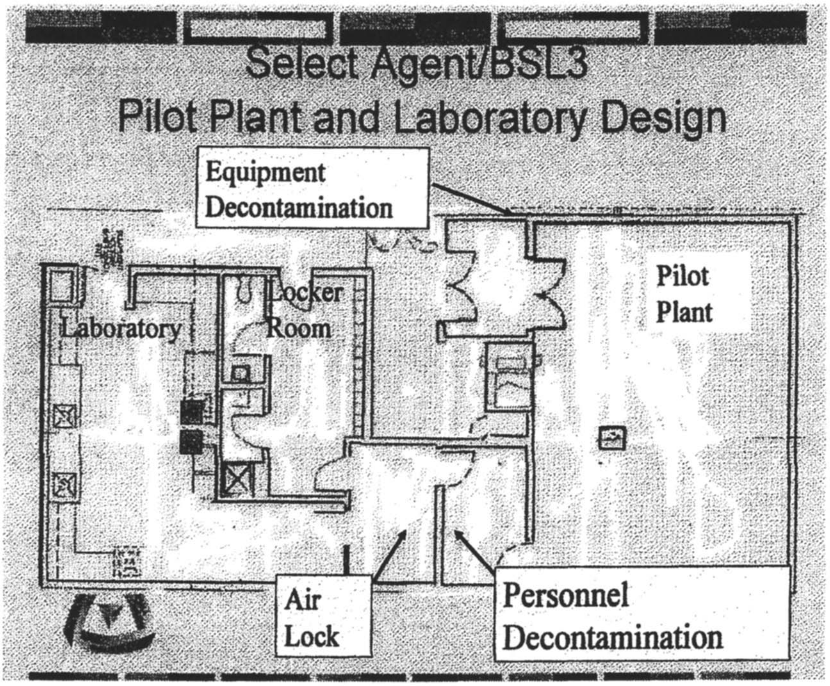
Select Agent/BSL3
Pilot Plant and Laboratory Design
Equipment Decontamination
Laboratory
Locker Room
Pilot Plant
Air Lock
Personnel Decontamination

*Figure 3. Food BSL Pilot Plant and Laboratory*

One of the elements that has evolved from our design research on this new pilot plant is that we need to have equipment decontamination, the capability of fully decontaminating the pilot plant and also verifying that it has been decontaminated each time equipment is moved in or out of the facility.

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A full decontamination of any facility is usually a difficult task and requires verification and validation. But, the ability to readily move equipment in and out of the pilot plant greatly increases the scope of the research that can be conducted in the facility. Also, effective personnel decontamination procedures are needed along with the requirements to meet all safety and security protocols of FDA and the Select Agent regulations.

There is also a need to upgrade the personal protective measures that go along with research on infectious pathogens. These include installing more biological safety cabinets and equipment in our laboratories. Also there are more legal requirements in terms of the storing, handling and shipping select agents. To ensure these legal requirements are met, security audits are necessary.

Scientists now also have to pass more stringent background and security checks. This causes delays with scientists newly hired to conduct counter-terrorism (CT) research. Also many young scientists with the desired expertise come from foreign countries that make security clearances difficult.

Finally, in terms of collaboration on CT research at NCFST, we are getting a mixed industry response. Some in the industry believe it is primarily a government responsibility to conduct CT research. Not all of the NCFST industry members are interested in collaborating in the kinds of CT research that the NCFST is conducting, even though what is being done applies to food safety as well. The NCFST is trying to connect food safety and food security so that it can apply the things learned in food security to food safety.

## Food as a Weapon of Terror

Certainly the contamination of food has always been an option for terrorists as evidenced by recent and ancient history. Tampering with food has the potential of causing large scale public health, economic and social disruptions. Food is something that each of us encounters on a personal basis daily and it is impossible to avoid consumption except on a relatively short-term basis. Added to this is the ready availability of a number of food pathogens and poisons that are cheaply and legally obtained. Finally in the open U.S. society, foods that are to be consumed are readily accessible and it is rather easy to imagine how one might access large quantities of food or food ingredients.

On the other hand, food presents certain unique difficulties to the potential terrorist. The first difficulty encountered is dosage and dosimetry. A food product may undergo a tremendous amount of mixing and combination with other food products before final distribution to the consumer. Any agent that was added might simply be diluted to the point that it is ineffective or inconsequential in terms of human health.

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Many pathogens or toxins may be inactivated by the food itself. The food product may be a hostile environment for a human pathogen that normally reproduces in living tissues. Likewise foods contain oxidizing and reducing agents, enzymes and other substances that may inactivate or substantially reduce the effectiveness of a poison. Finally, many foods are processed or cooked before eating such that a toxin or organism may be inactivated. At the very least their effect may be reduced by steps such as washing, formulation, drying, freezing, homogenization, separation, encapsulation, or other processing steps for foods.

Failed attempts to use biological weapons are often not well publicized and are sometimes known only after an extensive investigation and/or confessions by the "would be" perpetrators.

The Japanese cult Aum Shinrikyo attempted to disperse aerosols containing botulinal toxin on at least 3 occasions between 1990 and 1995 at multiple sites including downtown Tokyo and at US military installations in Japan (*20*). These attacks failed apparently because of faulty microbiological technique, deficient aerosol-generating equipment, or internal sabotage. This and other accounts of failed attacks would seem to indicate the use of biological agents as weapons is more difficult than might initially appear.

This point is further illustrated by the poisoning of baby food by ricin last month in California (*21*). The perpetrator apparently ground beans from an ornamental castor plant which contained tiny amounts of the poison ricin. The poison was not in a purified form and was not even present in sufficient amounts to cause the infants who ate the baby food to become ill.

If food is to be used as a vehicle for these weapons, then a more extensive knowledge of the agent, the food product and how it is processed, stored and distributed would seem to be required. While these may not seem to be major hurdles for some food professionals and scientists, they may indeed be major hurdles for those actually planning an attack involving the food supply.

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